MODULATION OF GRANULOCYTE APOPTOSIS BY GLUCOCORTICOIDS

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PhD Thesis.

The University of Edinburgh.

1997.

The work presented in this thesis was carried out solely by the author, unless otherwise stated, under the supervision of Dr Adriano Rossi and Professor Chris Haslett, Rayne Laboratory, University of Edinburgh.

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Acknowledgements

I would like to express my sincerest thanks to the following, for their practical help and support during this period of research.

Firstly, my uppermost gratitude extends to Professor Chris Haslett for providing me with the unique opportunity to undertake a PhD in such an exciting and novel field as 'Apoptosis'.

I would also like to extend my thanks to Dr. Adriano Rossi, to whom I am indebted to for his constant support, encouragement and friendship. He made many of the experiments possible and meticulously proof read this thesis, so once again 'thank you Adriano'.

A special thank you goes to Dr. Edwin Chilvers for his productive scientific discussions and precious time which inspired many of the experiments carried out.

I acknowledge Dr. Mark Giembycz for his hospitality during my visit to London and scientific expertise in the exasperating 'hunt' for PKA.

Thank you to many fellow laboratory colleagues, past and present, who have played a large part in making my three years in the Rayne Laboratory and time in Edinburgh both thoroughly enjoyable and extremely memorable. I would particularly like to mention Carol Ward, Joanna Murray, Elizabeth Kitchen and Katy Mecklenburgh for the many joys and lunches that we have shared, 'thanks girls'.

Finally, thank you to my best friend, Imad, for his endless encouragement and patience, and who has been there with me, from start to finish, through thick and thin, cider and gin.

To Mum and Dad

Abstract

Inflammation involving eosinophilic and neutrophilic granulocytes, which are closely related in ontogeny, has been associated with the pathogenesis of a variety of diseases such as asthma and other allergic conditions. It has been demonstrated *in vivo* that apoptosis is an important mechanism for the clearance of extravasated granulocytes from inflamed sites; leading to their phagocytosis by inflammatory macrophages, without exacerbating the inflammatory response. Eosinophils and neutrophils have been shown to undergo constitutive apoptosis *in vitro* and their rate of apoptosis can be modulated by a variety of growth factors and inflammatory mediators.

Glucocorticoids have long been successfully employed as potent anti-inflammatory agents, yet the means by which they exert their effects *in vivo* are poorly understood. The purpose of these studies was to determine the effects and mechanisms of action of glucocorticoids on eosinophil and neutrophil apoptosis and to examine the effects of these drugs on the phagocytosis of apoptotic granulocytes by macrophages.

We have demonstrated that dexamethasone exerts diametrically opposed effects on these two cell types; promoting eosinophil apoptosis and inhibiting neutrophil apoptosis. Similarly, elevation of $[Ca^{2+}]_i$ also differentially affects the rate of constitutive granulocyte apoptosis. In contrast, elevation of cAMP inhibits the rate of apoptosis in both granulocytes, whereas inhibition of PKC promotes the rate of granulocyte apoptosis. Moreover, inhibition of protein phosphatases inhibits the rate of granulocyte apoptosis at lower concentrations and promotes the rate of

granulocyte apoptosis at higher concentrations. Inhibition of the MAPK/ERK cascade inhibits the rate of constitutive eosinophil apoptosis, while having no effect upon the rate of constitutive neutrophil apoptosis. However, inhibition of this cascade selectively blocks the neutrophil survival-promoting effects of LPS, but exerts no effect on glucocorticoid-mediated inhibition of neutrophil apoptosis. Furthermore, we have demonstrated that dexamethasone mediates inhibition of neutrophil apoptosis by a PKA-dependent mechanism, whereas the pro-apoptotic effect of dexamethasone upon eosinophil apoptosis appears to be PKA-independent. Finally, we have shown that dexamethasone potentiates macrophage recognition and phagocytosis of apoptotic eosinophils and neutrophils, an event which may promote more efficient resolution of the inflammatory response.

Demonstration of the marked apoptosis-promoting effects of corticosteroids on eosinophil apoptosis together with the augmentation of macrophage clearance of apoptotic eosinophils, may in part explain the known beneficial therapeutic effects of corticosteroids on established 'eosinophilic' inflammatory diseases such as asthma. The opposite effect on neutrophil apoptosis may underlie the lower efficacy of these drugs in 'neutrophilic' inflammatory diseases such as ARDS. Further dissection of the intracellular mechanisms governing the divergent effects of corticosteroids on eosinophil and neutrophil apoptosis may lead to new therapeutic targets with which selective induction of apoptosis could be achieved.

Publications arising from thesis

Full publications

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Abbreviations

adenosine 5' triphosphate (ATP)

activating protein-1 (AP-1)

antibody (Ab)

adult respiratory distress syndrome (ARDS)

bovine serum albumin (BSA)

cAMP-response element binding protein (CREB)

dexamethasone (Dex)

ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)

fibronectin (Fn)

fura-2 acetoxymethyl ester (fura-2/AM)

glucocorticoid receptor (GR)

granulocyte-macrophage colony-stimulating factor (GM-CSF)

human bronchial epithelial cells (HBEC)

human serum albumin (HSA)

hydroxyicosatetraenoate (HETE)

immunoglobulin (Ig)

interleukin (IL)

intracellular calcium concentration $[Ca^{2+}]_i$

leukotriene (LT)

lipopolysaccharide (LPS)

MAP kinase kinase (MEK)

mitogen-activated protein kinase (MAPK)

N-formyl-methionyl-leucyl-phenylalanine (FMLP)

phorbol 12-myristate, 13-acetate (PMA)

phosphodiesterase (PDE)

platelet-activating factor (PAF)

prostaglandin (PG)

protein kinase A (PKA)

protein kinase C (PKC)

serum response factor (SRF)

sodium chloride (NaCl)

standard error of the mean (SEM)

thrombospondin (TSP)

vitronectin (Vn)

Contents

Chapter 1

Introduction

1.1 The inflammatory response

In the aftermath of physical trauma, particularly in response to bacterial or parasitic infection of tissues, there is a fairly stereotyped sequence of events involving vasodilatation and increased vascular permeability, leading to tissue oedema, which is often associated with a large leukocyte influx (Haslett, 1995). Finally, when all the invading organisms are phagocytosed by the recruited leukocytes, extravasated inflammatory cells such as eosinophils and neutrophils are cleared, thereby restoring normal tissue integrity and function in readiness for the next inflammatory insult. Clinically, these processes are well characterised, comprising of redness, heat, swelling, pain and loss or alteration of function. This innate response, known as 'inflammation', comprises of a co-ordinated network of mechanisms promoting wound repair (Baumann & Gauldie, 1994).

Paradoxically, when this same, normally beneficial, complex interplay of events occurs in a persistent or uncontrolled manner, the result is excessive tissue damage, often associated with the pathogenesis of a large number of inflammatory diseases. These include asthma, emphysema, chronic bronchitis, rheumatoid arthritis, and glomerulonephritis. Thus, elucidation of the mechanisms underlying the induction and resolution of the inflammatory response may help in developing an understanding of, and therapeutic approach to inflammation.

1.2 The eosinophil

The eosinophil plays a particularly important role in allergic inflammation and parasitic infections. Eosinophilia, characterised by an increased production of eosinophils in the bone marrow and the accumulation of eosinophils in tissues and blood, is specifically associated with a variety of diseases including asthma (Bousquet et al., 1990), chronic urticaria (Peters et al., 1983, Spry et al., 1985), allograft rejection (Lautenschlager et al., 1985), the hypereosinophilic syndrome (Fauci, 1982) and the eosinophil-myalgia syndrome (Hertzman et al., 1990).

Eosinophils originate from pluripotential stem cells in the bone marrow; the development and differentiation of which are specifically promoted by cytokines, such as GM-CSF, IL-3 and IL-5 (Sanderson, 1992). Eosinophils are mainly tissue-dwelling cells and are most abundant in tissues with an epithelial surface, such as the respiratory tract (Weller, 1994). Eosinophil tissue longevity is unknown but it is assumed to be at least several days, based on *in vitro* studies involving the induction of human eosinophil survival in culture over a week, upon addition of several peptide growth factors including GM-CSF, IL-3 and IL-5 (Tai et al., 1991) and in light of findings demonstrating that large numbers of eosinophils can be found in the tissues even when blood eosinophil counts are low (Weller, 1991). Blood counts reflect an intricate relationship between the number of cells leaving the bone marrow, marginating and then leaving the blood, with no significant recirculation of eosinophils back from the tissues to blood. The half-life of eosinophils in normal human blood is 18 h and is prolonged in eosinophilia, a finding that may be attributed to the limited number of sites through

which eosinophils can migrate into the tissues, causing cells to accumulate in the circulation.

1.2.1 Morphology

Eosinophils share some structural similarities with other granulocytes, but have bilobed nuclei and distinctive cytoplasmic granules (see *Appendix*). Eosinophilic granulocytes are slightly larger than neutrophils, having a mean diameter of 8 μm. The distinctive feature of mammalian eosinophils is the presence of characteristic secondary granules, with crystalloid cores. Eosinophils also contain two other types of granules: primary granules, which lack a crystalloid core and are characteristically seen in eosinophilic promyelocytes (Bainton & Farquhar, 1970), and the so-called small granules, which reportedly contain aryl-sulphatase and other enzymes (Parmley & Spicer, 1974). Prominent tubulovesicular structures are sometimes identified as a fourth population of granules (microgranules) (Parmley & Spicer, 1974). Other cytoplasmic structures of the eosinophil, include lipid bodies, which are non-membrane-bound, lipid-rich inclusions found in many cell types and are often misidentified as granules (Weller, 1991). The functions of the lipid bodies have not been fully elucidated but they do serve as intracellular sites of arachidonic acid storage and metabolism (Bozza et al., 1996).

1.2.2 Intracellular constituents

Secondary granules contain lysosomal hydrolases as well as most of the cationic proteins unique to eosinophils. The crystalloid core of the granule is composed of major basic protein (MBP) and the matrix surrounding the core contains three well-characterised cationic proteins, namely eosinophil cationic protein (ECP), eosinophil-derived neurotoxin

(EDN) and eosinophil peroxidase (EPO) (Peters et al., 1986, Egesten et al., 1986). The functional properties of these cationic proteins have been well studied and are summarised overleaf. MBP has no recognised enzymatic activity, but is toxic to helminthic parasites, tumour cells and host cells (Butterworth et al., 1979, Gleich & Adolphson, 1986). ECP is less abundant than MBP in granules, with bactericidal and helminthotoxic activities. EDN is a potent neurotoxin but is a relatively weak helminthotoxin. Finally, EPO, which in the presence of hydrogen peroxide is able to oxidise halides to form highly reactive hypohalous acids. Thus, under these conditions EPO is toxic to helminthic and protozoan parasites, bacteria, tumour cells and host cells (Venge, 1993). Another distinctive protein found in the primary granules of human eosinophils, forms Charcot-Leyden crystals, which constitutes a hydrophobic protein with lysophospholipase activity.

1.2.3 Mediator release

In addition to the toxic cationic proteins, upon appropriate stimulation, eosinophils synthesise and secrete a number of inflammatory mediators including lipids, such as platelet-activating factor (PAF) and leukotriene C_4 (LTC_4) and cytokines, including transforming growth factor alpha (TNF-α), IL-1, IL-3, IL-5 and GM-CSF (Weller & Austen, 1983, Kita et al., 1991a, Moqbel et al., 1994). Eosinophils are also capable of generating two potentially toxic oxygen metabolites, the superoxide radical anion, O_2^- and H_2O_2.

1.2.4 Mechanisms of eosinophil recruitment at sites of inflammation

Following tissue injury, eosinophils migrate and emigrate into tissues through post-capillary venules where slow blood flow allows loose

attachments to endothelial cells (Weller, 1991). Inflammatory mediators, such as C5a, leukotriene B_4 (LTB_4), IL-8 and eotaxin, are generated in response to an appropriate stimulus and result in cellular activation. Eosinophils express on their surface either selectins (E-, L- and P-selectin), or selectin ligands, which primarily mediate tethering of the circulating eosinophil and allow rolling on the endothelial surface of post-capillary venules. Locally released mediators bind to specific eosinophil receptors leading to increased expression of eosinophil surface integrins, in particular β_1 and β_2 integrins. These integrins bind to their ligands on the endothelial cell for example, ICAM-1, VCAM-1, and strengthen the eosinophil-endothelial cell adhesion, resulting in firm adhesion. The eosinophils can then traverse the endothelial cell layer of the blood vessel wall into the extravascular tissue space, travelling towards the inflammatory focus along a chemotactic gradient and are finally immobilised in the locality of the infection. This model describes the generalised mechanisms underlying recruitment of both eosinophilic and neutrophilic inflammatory cells, at post-capillary venules of the systemic microcirculation (Carlos & Harlan, 1994, Springer, 1994). However, selective recruitment of the different inflammatory cell types is achieved by the existence of multiple steps that are specific for each granulocyte type. For example, generation of specific inflammatory mediators determines which type of inflammatory cell is recruited. In this regard, the C-C chemokines RANTES, monocyte chemotactic protein (MCP)-3 and eotaxin stimulate eosinophils *in vitro* (but not neutrophils) (Baggiolini & Dahinden, 1994, Schall & Bacon, 1994, Kita & Gleich, 1996) and eotaxin (Griffiths-Johnson et al., 1993, Jose et al., 1994) appears to cause a specific eosinophil accumulation *in vivo*. Thus, these chemokines appear to be key mediators in the induction of a tissue

eosinophilia. In contrast, the C-X-C chemokine IL-8 is a potent neutrophil chemoattractant (Teran et al., 1993). The expression of different adhesion molecules may also allow the specific accumulation of one cell in relation to another. Expression of the β_1 integrin VLA-4 and the β_7 integrin $\alpha_4\beta_7$ by eosinophils but not neutrophils, suggests that through binding to VCAM-1 these adhesion pathways allow selective migration of eosinophils (Wardlaw et al., 1994, Walsh, 1997). The recruitment of granulocytes therefore depends upon the complex interaction between the nature of the injurious stimulus, the inflammatory mediators generated, the adhesion molecules expressed and the physiology of the vasculature.

1.2.5 Functions

Over the years, there has been considerable debate regarding the role of eosinophils in inflammatory diseases such as asthma. It is now known that eosinophils can have roles that are both beneficial and detrimental to the host. These granulocytes have a specialised role in host defence against large organisms that are not able to be phagocytosed, most notably the multicellular helminthic parasites (Butterworth, 1984). Interestingly, although eosinophils are capable of phagocytosing and destroying small microbes and bacteria *in vitro*, this does not form part of their major role in host defence *in vivo*. Moreover, eosinophils cannot effectively defend against bacterial infections when neutrophil function is deficient, as is apparent in leukocyte adhesion deficiency syndrome (Anderson et al., 1985). The eosinophil's armamentarium of noxious substances makes these granulocytes particularly toxic to helminths, in particular since MBP and ECP, which are the first cationic proteins to be deposited after binding to the surface of the parasite, are potent helminthotoxins and

moreover, since EPO generates hypohalous acids that also kills parasites such as *Schistoma mansoni* (Jong et al., 1981). Finally, eosinophil oxidative products also mediate helminthotoxicity (Butterworth, 1984). Uncontrolled release of the eosinophil-derived superoxide anions and cationic proteins active in host defence, can prove detrimental to the host. Thus, the eosinophil by virtue of its cytotoxic capabilities can cause many of the adverse effects associated with an allergic reaction, such as epithelial injury in the lungs of asthmatics (Gleich et al., 1988).

1.3 The neutrophil

The neutrophil is specifically associated with diseases such as rheumatoid arthritis (Brown, 1988), however this granulocyte has also been implicated in a variety of pulmonary inflammatory diseases, such as adult respiratory distress syndrome (ARDS) and neonate (Weiland et al., 1986). The size of its contribution in human asthma and other allergies remains uncertain. The eosinophilic granulocyte is thought to be more critically involved in the pathogenesis of asthma however, the prominent role of the neutrophil is becoming more apparent with the abundance of neutrophilic granulocytes reported in some cases of fatal human asthma (Sur et al., 1993).

Neutrophils are the most abundant leukocytes in the blood, constituting approximately half of the white cell population and originate from myeloid precursors in the bone marrow. Constitutive granulocytopoesis is under the control of general growth factors such as IL-3, GM-CSF and G-CSF. Neutrophils are short-lived cells, with a circulating half-life of

only 6 h and therefore need to be continuously replaced in order to maintain a stable circulating neutrophil population.

1.3.1 Morphology

Neutrophils have a mean diameter of 7 μm, a multilobed nucleus and a large number of intracellular granules (see *Appendix*). The traditional classification of neutrophil granules as peroxidase-positive (azurophil or primary) and peroxidase-negative (specific or secondary) has now been revised, describing four essentially different types of intracellular granules and vesicles (Borregaard et al., 1993), which are summarised below. Secretory vesicles, have been identified as a highly mobilisable light membrane structure, containing alkaline phosphatase (Borregaard et al., 1990). It has now been established that the secretory vesicle membrane also contains the adhesion protein CD11b/CD18 (Mac-1), which is essential for integrin-mediated neutrophil adhesion (Calafat et al., 1993), the receptor for the chemotactic tripeptide, FMLP (Jesaitis et al., 1982) and the membrane component of NADPH oxidase, cytochrome b_{558} (Calafat et al., 1993). Specific (or secondary) granules, are spherical in shape and are defined by their lactoferrin content. They are also an important source of cytochrome b_{558} (Borregaard & Tauber, 1984), adhesion receptors (Bainton et al., 1987) and FMLP receptors (Fletcher & Gallin, 1983). Gelatinase (or tertiary) granules, are smaller than specific granules and constitute approximately 25% of all peroxidase-negative granules. They are principally composed of the protease gelatinase, which may be involved in digesting the vascular basement membrane to allow neutrophil extravasation (Peppin & Weiss, 1986). Finally, azurophil (peroxidase-positive) granules, generally contain agents that are released into the phagocytic vacuole at the end of the phagocytic process. These agents include digestive proteinases and microbicidal

proteins, including defensins. Moreover, the membrane of azurophil granules has not been shown to contain any receptors or adhesion molecules of importance for interaction of the neutrophil with its environment. Of particular significance, is the presence of myeloperoxidase in azurophil granules, which is required for full function of the oxygen-dependent bactericidal system in neutrophils. Thus, azurophil granules, in particular, contain many noxious agents effective in bacterial killing, which if inappropriately released would be highly detrimental to host tissue.

1.3.2 Mediator release

As well as a variety of cytoplasmic and granule associated enzymes, neutrophils are also able to synthesize and release a number of inflammatory mediators including lipids, such as PAF, LTB_4 and 5-HETE and cytokines, including IL-1α and β, TNF-α, IL-6, IFNα, GM-CSF and IL-8 (McColl & Showell, 1994).

1.3.3 Functions

Neutrophils play important roles in host defence against all classes of infectious agents and constitute the first line of defence against nonself substances. Their major role is to phagocytose and destroy pathogens, such as bacteria, fungi and protozoa. With many microbes, however, neutrophil defences are ineffective in the absence of opsonins and various agents that amplify the cytotoxic response. Thus, bacteria and other foreign particles are phagocytosed most effectively when coated with opsonizing IgG, promoting recognition by the Fc receptors (FcR) of the neutrophil.

Although neutrophils are essential to host defence, it is now clear that neutrophils are involved in several aspects of the inflammatory response and have been implicated in the pathology of many chronic inflammatory conditions (Weiss, 1989). Host tissue damage may occur by a variety of mechanisms including release of microbicidal products into the extracellular milieu during the killing of microbes and/or failure to terminate acute inflammatory responses. However, under physiological conditions, a number of mechanisms exist to limit the destructive capacity of inflammatory cells and allow resolution to occur. These include a reduction in the effective concentration of pro-inflammatory stimuli; an increase in anti-inflammatory mediators; decreased cellular responsiveness and ultimately death by apoptosis and removal of the cell itself.

1.4 Apoptosis

Before the seminal work of Wyllie and colleagues (Kerr et al., 1972, Wyllie et al., 1980), who described a physiological mode of cell death termed apoptosis, it was widely believed that the usual fate of cells was to die by necrosis. This 'accidental' form of cell death refers to the morphology most often seen when cells die from severe and sudden injury, such as ischaemia or physical or chemical trauma. The distinctive morphology and biochemical characteristics associated with necrosis (Schwartzman & Cidlowski, 1993), ultimately lead to organelle dissolution and rupture of the plasma membrane, allowing release of the cytotoxic cellular contents into the surrounding milieu. Thus, in contrast to apoptosis, necrosis usually provokes an inflammatory response.

Apoptosis is a widespread phenomenon that plays a crucial role in a myriad of physiological and pathological processes (Ellis et al., 1991). It is, for example, essential in many aspects of normal development and is required for homeostasis, maintaining correct numbers of cells in the body by balancing cell production with cell death. Moreover, apoptosis represents a natural form of cell death that is now associated with the resolution of the inflammatory response (Haslett et al., 1994). In addition, to the beneficial effects of apoptosis, the inappropriate activation of apoptosis may cause or contribute to a variety of diseases including acquired immunodeficiency syndrome (AIDS) and neurodegenerative diseases (Savill, 1994).

Apoptosis has been associated with specific and characteristic morphological and biochemical changes, which may be indicative of a common underlying series of molecular mechanisms. Importantly, apoptotic cells remain functionally intact, retaining their cytoplasmic granules and maintaining plasma membrane integrity, as assessed by vital dye exclusion (e.g., trypan blue). Moreover, apoptosis leads to swift recognition, uptake and degradation of intact cells by phagocytes, without release of noxious contents, thus protecting host tissue against potentially inflammatory stimuli (figure 1.1). The distinctive morphology of apoptosis has been described in detail from observations using light and electron microscopy (Wyllie et al., 1980). Briefly, characteristic morphological changes include, a reduction in cellular volume, condensation of chromatin around the periphery of the nucleus and eventual dissolution of the entire cell into small membrane-bound, smooth-surfaced, spherical structures known as apoptotic-bodies, although apoptotic bodies are rarely formed during granulocyte apoptosis.

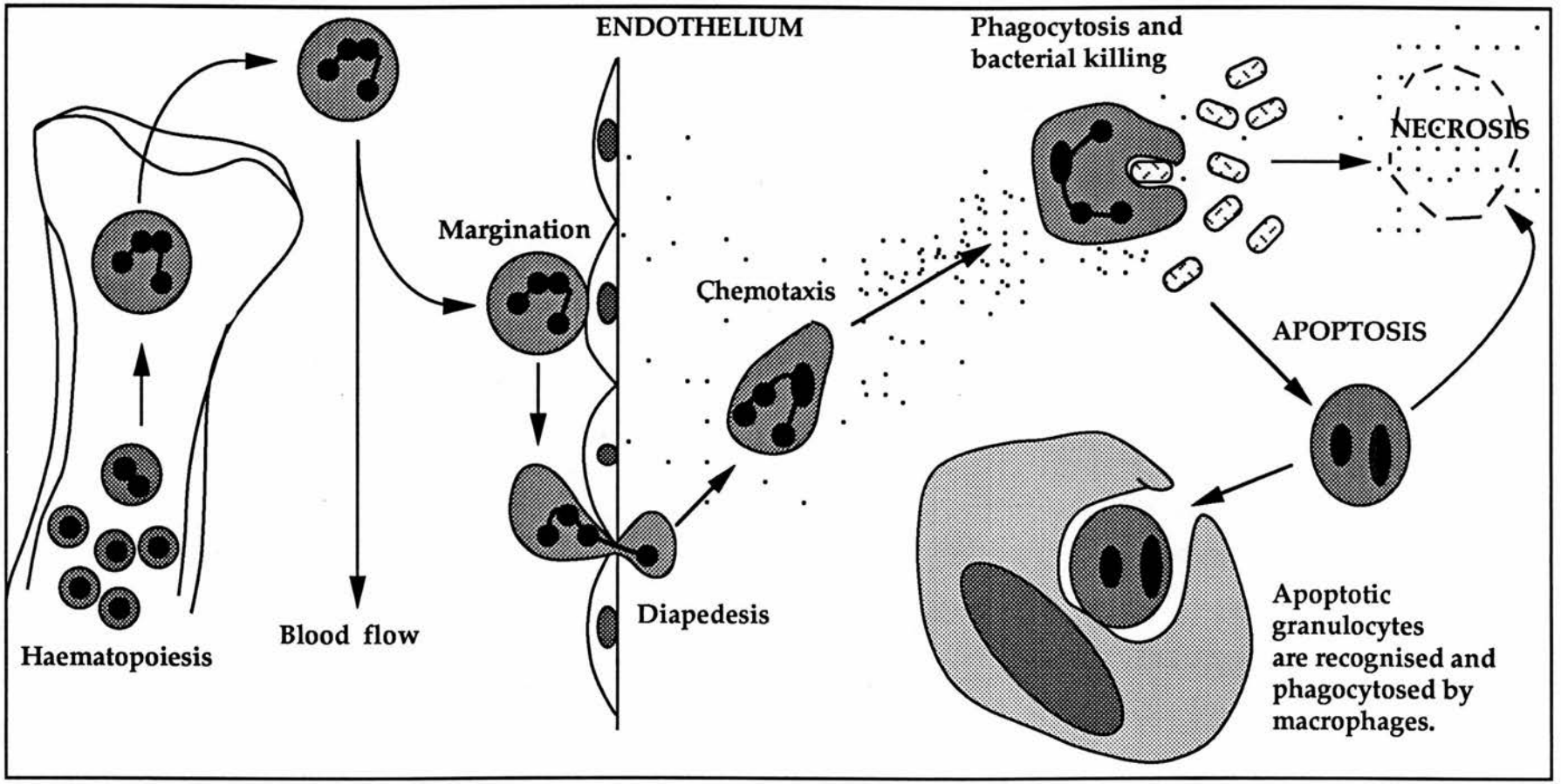

Figure 1.1 The life cycle and role of eosinophilic and neutrophilic granulocytes.
Granulocytes produced during haematopoiesis in the bone marrow are released into the circulation, where they adhere to and migrate through the endothelium. Moving by chemotaxis through the tissues to the site of infection, they carry out their roles of host defence. Senescent granulocytes undergo apoptosis and are phagocytosed by local macrophages. Granulocytes that are not recognised by macrophages die by necrosis, exacerbating the inflammatory response.

This is accompanied by the fragmentation of DNA, which forms a ladder pattern of regular subunits when subjected to agarose gel electrophoresis. Because of its near universality, internucleosomal DNA degradation is considered a diagnostic hallmark of cells undergoing apoptosis. Apoptosis can also be assessed by various other methods, including propidium iodide (PI) staining, which involves changes in dye binding to nuclear material (Nicoletti et al., 1991) and by measuring cell surface changes. With regard to this latter method of evaluating apoptosis, most apoptotic cells express phosphatidylserine molecules on the external surface of their plasma membranes. This provides the acquired property of binding annexin V, which is a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine. Thus, the almost universal exposure of phosphatidylserine on apoptotic cells has now been shown to serve as a reliable diagnostic for the occurrence of apoptosis *per se* (Koopman et al., 1994, Homburg et al., 1995). Moreover, in specific cells such as the neutrophil, shedding of cell surface FcγRIII (CD16) has been associated with the appearance of apoptotic morphology and hence levels of CD16 expression can be used to define non-apoptotic and apoptotic neutrophils (Dransfield et al., 1994). It has also been shown that during apoptosis, there is a marked loss of a number of neutrophil functions, including chemotaxis, phagocytosis and secretion of granule enzymes after deliberate external neutrophil stimulation (Whyte et al., 1993a), thereby rendering this cell functionally isolated from the surrounding environment.

1.4.1 Clearance of apoptotic cells

It has now been demonstrated both *in vitro* (Haslett et al., 1987, Stern et al., 1992) and *in vivo* (Savill et al., 1989a, Woolley et al., 1996) that

apoptotic granulocytes can be cleared by macrophages without release of pro-inflammatory mediators, such as thromboxane B_2 and cytokines, which are normally released during phagocytosis, exemplifying the tissue-injury limiting properties of this clearance mechanism (Meagher et al., 1992, Stern et al., 1992).

Macrophages originate from pluripotential stem cells in the bone marrow and circulate in the bloodstream as mature monocytes which, under normal steady-state conditions, migrate into tissues and body cavities where they differentiate into macrophages. They have a central role in host defence, by nature of their noxious secretory substances and are particularly important for phagocytosing invading organisms prior to leukocyte recruitment.

1.4.2 Regulation of apoptosis

The highly organised removal of 'unwanted' cells by apoptosis strongly suggests that the apoptotic process is subject to careful regulation. However, the study of apoptosis and its control, is complicated by the fact that diverse stimuli may be responsible for initiating the onset of the apoptotic programme in different cell types. Hence, signals producing apoptosis in one cell type may have differing effects on other cell types; many examples of which will be discussed in this thesis.

The onset of apoptosis in both eosinophils and neutrophils can be delayed by haematopoietins and inflammatory mediators (Stern et al., 1992, Lee et al., 1993); the specific details of which are discussed in Chapter 3. However, comparatively speaking, very little is known about the precise controls and triggers of granulocyte apoptosis, particularly in

contrast to, for example, apoptosis in thymocytes, which can be induced by a variety of different mechanisms. Glucocorticoid treatment, irradiation, Fas ligand and TNF-α are all pro-apoptotic signals in the thymocyte (Schwartz & Osborne, 1993, Suda & Nagata, 1994). It has recently been demonstrated that anti-Fas activating antibodies or Fas ligand promote eosinophil and neutrophil apoptosis, an effect prevented by anti-Fas blocking antibodies (Matsumoto et al., 1995, Liles et al., 1996). Moreover, hypoxic conditions, which are found in chronically inflamed sites, inhibit neutrophil apoptosis, whereas in some cell lines, apoptosis is induced (Hannah et al., 1995). Mixed genetic control of apoptosis by individual genes has also been demonstrated by various cells. Some cells expressing the c-myc proto-oncogene product, have been shown to undergo apoptosis readily, whereas other cells expressing the oncogene product bcl-2 are protected from undergoing apoptosis (Schwartz & Osborne, 1993). However, the significance of such molecular control on eosinophil and neutrophil apoptosis is unknown.

1.5 Glucocorticoids

Glucocorticoids are highly effective in the control of many inflammatory and immune diseases and have been employed therapeutically for many years. Inhaled steroids have now become first-line therapy for the treatment of chronic asthma, yet the mechanisms by which they exert their effects *in vivo* are still poorly understood (Goulding & Guyre, 1993). Further studies elucidating the underlying anti-inflammatory mechanisms employed by these agents may offer attractive therapeutic alternatives, with fewer clinical side-effects.

1.5.1 Glucocorticoid receptors

Glucocorticoids diffuse passively through the cell membrane into the cytoplasm, where they bind reversibly with high affinity and specificity, to soluble receptor proteins known as glucocorticoid receptors (GRs). The GR is a member of the steroid hormone receptor supergene family, which includes cytosolic receptors for other steroid hormones such as progesterone. Moreover, it has been established that there are two receptor isoforms of the human GR, namely hGRα and hGRβ, which originate from the same gene by alternative splicing (Hollenberg et al., 1985, Encio & Detera-Wadleigh, 1991). These isoforms have been detected in a variety of tissues, including cells from the lungs (Ballard & Ballard, 1974) and in inflammatory cells such as eosinophils and neutrophils (Peterson et al., 1981) and macrophages (Werb et al., 1978). Moreover, it has been demonstrated that saturable glucocorticoid binding in human eosinophils is similar to that observed in neutrophils and the affinity of the GR (Kd = 15.3 ± 0.6 nM in the eosinophil and 17.7 ± 0.8 nM in the neutrophil) indicates that it is capable of mediating effects at physiological hormonal concentrations (Peterson et al., 1981).

The structural organisation of the GR is characterised by a short and highly conserved cysteine-rich central region, constituting the DNA-binding domain; a relatively well-conserved carboxy-terminal domain, important for both hormone binding and transactivation and a variable amino-terminal region containing the transactivation domains responsible for gene activation. The GR is a phosphoprotein (Housley & Pratt, 1983), being phosphorylated predominantly on serine residues at the N-terminal, but the definitive role of phosphorylation in steroid actions is unknown (Muller & Renkawitz, 1991).

In the absence of ligand, the GR is part of a heteromeric complex consisting of one receptor molecule, two molecules of 90 kDa heat shock protein (hsp 90) and one molecule each of hsp 70, hsp 57 and hsp 23 (Hutchison et al., 1993, Czar et al., 1994). The hsp molecules act as a 'molecular chaperone', preventing the unoccupied GR from localising to the nuclear compartment. As depicted in figure 1.2, hormone-binding leads to a conformational change in the GR molecule, allowing it to dissociate from the hsp complex, homodimerize with another hormone-activated receptor molecule and translocate to the nucleus, where it exhibits DNA binding ability and interacts with a wide range of nuclear targets. Activated GRs have been shown to bind to DNA at consensus sites termed glucocorticoid response elements (GREs) and negative GREs (nGREs), in the regulatory regions of glucocorticoid target genes, resulting in transcriptional activation and repression, respectively (Yamamoto, 1985). Thus, glucocorticoids are known to both up- and down-regulate the expression of specific proteins (Maroder et al., 1993) and it is this protein regulation which is ultimately thought to bring about altered cell function and expression of glucocorticoid activity. The GR is therefore a signal transduction protein that transduces the hormone signal to a transcriptional response at the level of specific target genes, which are expressed in a cell-specific manner.

1.5.2 Receptor interaction with transcription factors

GRs have the ability to interact with other transcription factors and this may be an important determinant of steroid responsiveness underlying the anti-inflammatory actions of glucocorticoids (Schule & Evans, 1991, Ponta et al., 1992, Cato & Wade, 1996). Studies show that direct protein-protein interactions between the components of AP-1 or NF-*k*B and GRs

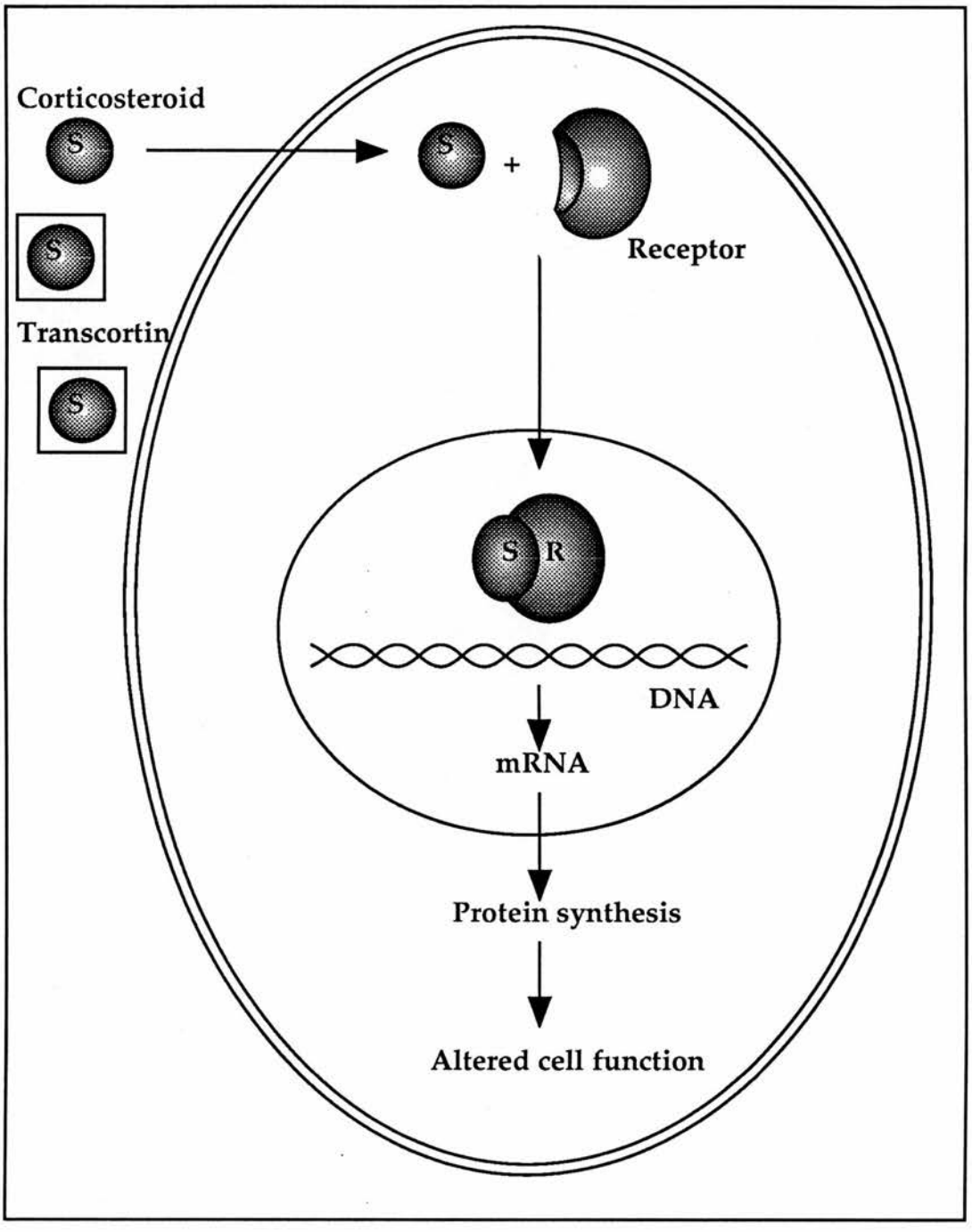

Figure 1.2 Simplified general mechanism of steroid action.
Circulating steroids are complexed to binding proteins such as transcortin and albumin. However, a small percentage of exogenous synthetic corticosteroids circulate in an unbound and active state. These free steroid molecules (S) enter cells, combine with specific receptors (R), and move to the nucleus where new mRNA is synthesised. The mRNA initiates new cytosolic protein synthesis, which may alter cellular function.

may prevent DNA binding and/or transactivation by these pro-inflammatory transcription factors, thereby partly accounting for the inflammation suppressive properties of glucocorticoids (Adcock et al., 1994a, Mukaida et al., 1994, Ray & Prefontaine, 1994). For example, in human lung TNF-α and phorbol esters reportedly increase AP-1 and NF-*k*B binding to DNA, and this is inhibited by glucocorticoids (Adcock et al., 1994a; 1994b). Moreover, an additional mechanism of repression by glucocorticoids on NF-*k*B-mediated transcription has been demonstrated, involving a rapid induction of I*k*Bα mRNA and protein synthesis, following steroid treatment (Auphan et al., 1995). Newly synthesised I*k*Bα interacts with mRNA heterodimers thereby inhibiting NF-*k*B DNA binding and transcriptional activation by cytokines. However, Adcock and colleagues have recently shown that IL-1β induces GM-CSF release in human lung epithelial-like (A549) cells via a NF-*k*B-dependent mechanism and repression occurred at concentrations of dexamethasone that were shown not to induce I*k*B (Adcock et al., 1997). Collectively, these findings suggest that the induction of I*k*B does not represent a universal anti-inflammatory mechanism underlying glucocorticoid action.

GRs may interact with other transcription factors, such as CREB. There is evidence that β-adrenoceptors, via formation of cyclic AMP and activation of PKA, result in the activation of the transcription factor CREB, that binds to a cyclic AMP response element (CRE) on genes (Yamamoto et al., 1988). β-Adrenoceptor agonists increase CRE binding in human lung *in vitro* and at the same time reduce GRE binding, suggesting that there may be a protein-protein interaction between CREB and GRs within the nucleus (Peters et al., 1993). Similar interactions

have been demonstrated in a hepatoma cell line (Imai et al., 1993) and in human pulmonary epithelial cells (Stevens et al., 1995).

1.5.3 Anti-inflammatory mechanisms of glucocorticoids

The anti-inflammatory mechanisms employed by glucocorticoids in controlling inflammation may be effective by regulating several aspects of the inflammatory process through increasing or decreasing gene transcription. Possible mechanisms particularly germane to the *results* presented in this thesis are introduced below.

1.5.3.1 Cytokines

Cytokines play an important role in chronic inflammation and it has been reported that steroids have potent inhibitory effects on cytokine-mediated inflammation. Steroids inhibit the transcription of several pro-inflammatory cytokines, including TNF-α, GM-CSF, IL-1, IL-3, IL-4, IL-5, IL-6 and IL-8 (Guyre et al., 1988, Rolfe et al., 1992). These effects may be mediated directly via interaction of GRs with nGREs in the upstream promoter sequence of the cytokine gene, resulting in reduced gene transcription. However, since there is no apparent nGRE consensus sequence in the upstream promoter region of many cytokines, such as IL-2, IL-8 and RANTES, it has been suggested that glucocorticoids also inhibit transcription indirectly via interference with other transcription factors. Thus, glucocorticoid-mediated inhibition of IL-2 gene transcription in T lymphocytes is predominantly regulated by a cell-specific transcription factor, nuclear factor of activated T cells (NF-AT), which is activated in the cytoplasm on T cell receptor stimulation via calcineurin (Thomson & Starzl, 1993). The nuclear factor AP-1 also appears to be necessary for increased activation, forming a transcriptional

complex with NF-AT by direct binding (Northrop et al., 1993). Glucocorticoids inhibit IL-2 gene transcription indirectly by binding to AP-1, thereby preventing increased transcription due to NF-AT (Paliogianni et al., 1993). Transcription of IL-8 and RANTES is inhibited by glucocorticoids in a similar manner. Negative regulation of IL-8 is mediated by the interaction of glucocorticoids with NF-*k*B (Mukaida et al., 1994), while the transcription of RANTES is inhibited by the interaction of glucocorticoids with both NF-*k*B and AP-1 (Nelson et al., 1993).

In addition to blocking the synthesis of cytokines, steroids may also inhibit the synthesis of certain cytokine receptors, as has been demonstrated with the IL-2 receptor (Grabstein et al., 1986). Interestingly in neutrophils, it has been reported that dexamethasone can induce synthesis of the type II receptor (a decoy molecule for IL-1); an effect which has been suggested to contribute to the anti-inflammatory activities of dexamethasone (Muzio et al., 1994). Moreover, steroids have also been reported to counteract cytokine-induced activation of certain transcription factors, such as TNF-α-mediated activation of AP-1 and NF-*k*B (Adcock et al., 1994a).

1.5.3.2 Inhibitory proteins

Glucocorticoids may have an inhibitory effect on inflammation by increasing the synthesis of anti-inflammatory proteins, such as lipocortin-1 (Flower & Rothwell, 1994). This 37 kDa protein inhibits the action of phospholipase A_2 and may therefore inhibit the production of arachidonic metabolites, such as leukotrienes, prostaglandins and PAF. Thus, production of lipocortin-1 would act to limit the pro-inflammatory potential of the above lipid mediators. Steroids induce the formation of

lipocortin-1 in several cells, including rat and human leukocytes (Goulding et al., 1990, Peers et al., 1993). Moreover, it has been reported that recombinant lipocortin-1 has acute anti-inflammatory properties and inhibits the release of pro-inflammatory mediators such as thromboxane from guinea-pig isolated perfused lung (Cirino et al., 1987).

Steroid-induced production of lipocortin-1 was one of the first proposed anti-inflammatory mechanisms of action of the glucocorticoids (Flower & Rothwell, 1994). However, it is now apparent that lipocortin-1 has rather non-specific effects (Davidson & Dennis, 1989) and moreover, glucocorticoids do not induce expression of lipocortin-1 in all cell types (Bronnegard et al., 1988). Thus, while production of lipocortin-1 may account for several of the pharmacological actions of glucocorticoids, it does not underlie a universal anti-inflammatory mechanism of steroid action.

1.5.3.3 Effect on cell function

Steroids may have direct inhibitory actions on several inflammatory cells implicated in inflammatory diseases such as asthma (Barnes et al., 1995). Inhibition of inflammatory mediator release from eosinophils, macrophages and T lymphocytes has been a reported cellular effect of glucocorticoids. One of the best described actions attributed to steroids in asthma is a reduction in the number of circulating eosinophils after administration of corticosteroids, an observation which has been suggested may reflect an action on eosinophil production in the bone marrow (Barnes, 1995a). Although corticosteroids are known to reduce the neutrophil influx to an inflammatory site, the direct effect of these agents on neutrophil function remains ill defined.

1.5.3.4 Effect on cell survival

The survival of certain inflammatory cells, such as eosinophils and thymocytes is markedly reduced after exposure to steroids. It is now well documented that eosinophil survival is dependent on the presence of such cytokines as IL-5 and GM-CSF and that exposure to steroids inhibits the survival-promoting effects of these cytokines, leading to apoptosis (Lamas et al., 1991, Owens et al., 1991, Wallen et al., 1991, Hallsworth et al., 1992). The mechanisms by which the inhibition of eosinophil survival may occur have not been fully defined but increased expression of specific endonucleases has been suggested as being relevant to the action of steroids on inflammatory cell survival (Compton & Cidlowski, 1987, Owens et al., 1991). Thus, the main focus of my thesis has been to investigate the mechanisms underlying the effect of corticosteroids on granulocyte apoptosis.

1.6 Signal transduction

Between the signal that initiates the process of apoptosis and the genes encoding the machinery that kills the cell, are signal transduction pathways. Several different signal transduction systems have been associated with the regulation of apoptosis and the final response varies with cell type. The details appertaining to the modulation of apoptosis by specific signal transduction pathways will be discussed in the chapters following, however the classical pathways are introduced below.

1.6.1 Calcium (Ca^{2+}) and Protein kinase C (PKC)

Elucidation of the role of Ca^{2+} as an intracellular messenger, began over one hundred years ago with the observation by Ringer (1881) that the contraction of cardiac muscle requires the presence of extracellular Ca^{2+}. Changes in the concentration of free Ca^{2+} in the cytoplasmic space ($[Ca^{2+}]_i$) have subsequently been shown to act as an intracellular

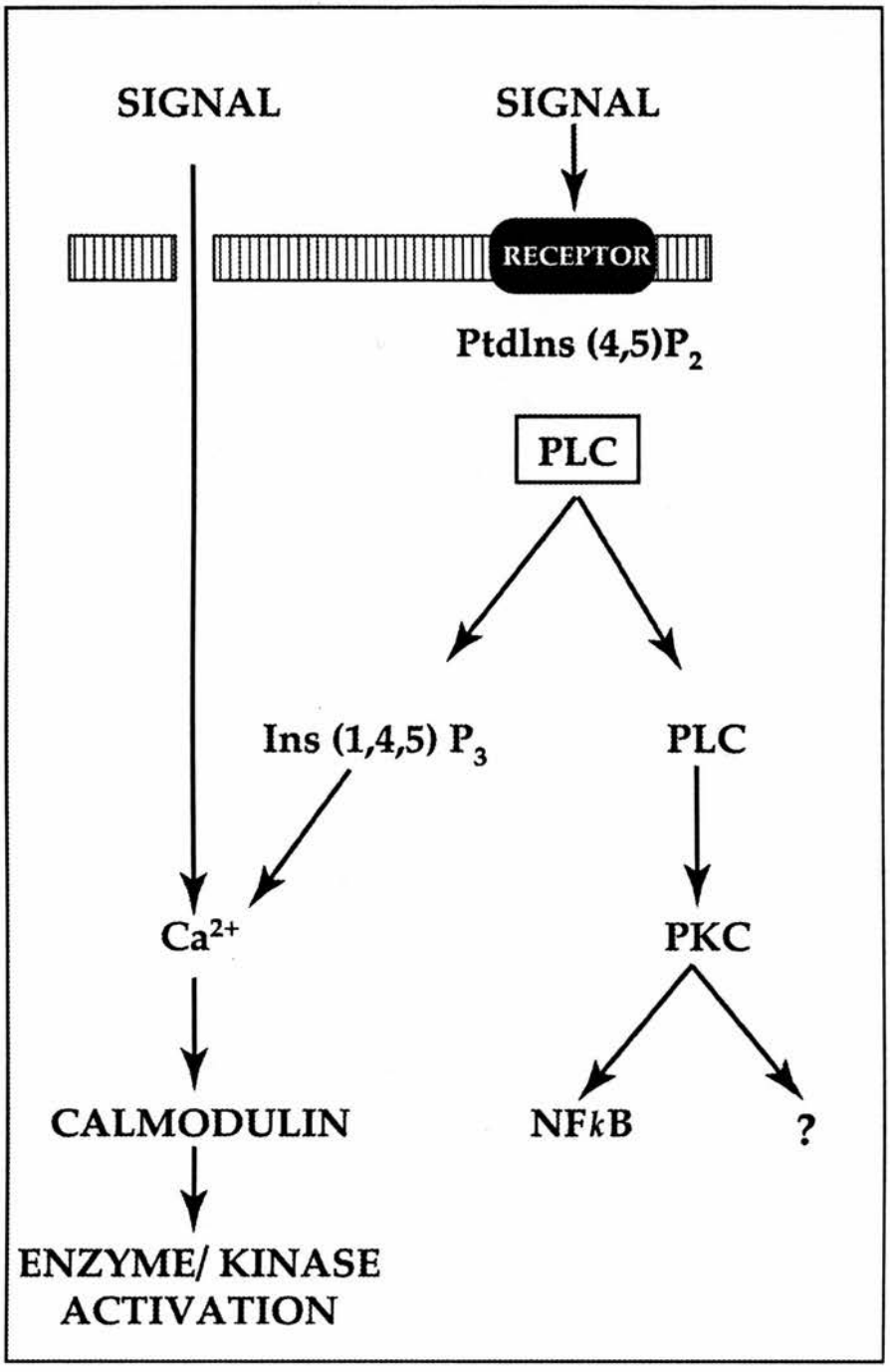

Figure 1.3 Schematic diagram illustrating Ca^{2+} and PKC signal transduction pathways.

messenger in most cells, ranging from bacteria to specialised neurones (Clapham, 1995). As illustrated in figure 1.3, stimulation by a hormone or neurotransmitter increases the $[Ca^{2+}]$ in the cytosol as calcium channels open in the plasma membrane or as Ca^{2+} is released from intracellular stores, such as the endoplasmic or sarcoplasmic reticulum and mitochondria. When the concentration rises, Ca^{2+} binding proteins in the cytosol, such as the specific receptor, calmodulin, attach to Ca^{2+} and the Ca^{2+}-protein complexes then interact with other proteins in the cell to alter their functions.

Metabolism of inositol phospholipids represents another mechanism by which the $[Ca^{2+}]_i$ is elevated. The initial signal is transduced via a GTP-binding protein, which in turn activates phospholipase C (PLC). This leads to the hydrolysis of phosphatidylinositol (4,5)-bisphosphate [PtdIns $(4,5)P_2$] to form the second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate [Ins $(1,4,5)P_3$], which constitute an interacting pair of signals that activate the Ca^{2+}- and phospholipid-dependent enzyme PKC (Takai et al., 1979, Nishizuka, 1984) and liberate intracellular sequestered Ca^{2+}, respectively. The effects of Ins $(1,4,5)P_3$ can be mimicked *in vitro* by pharmacological agents such as (1) thapsigargin, which increases cytosolic Ca^{2+} by inhibiting the ATP-dependent endoplasmic reticulum Ca^{2+} pump, thereby promoting Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} by a capacitative mechanism (Thastrup et al., 1989) and (2) Ca^{2+} ionophores such as A23187, which increase intracellular Ca^{2+} by facilitating Ca^{2+} influx across the plasma membrane (Reed & Lardy, 1972). In neutrophils, for example, combined treatment of cells with Ca^{2+} ionophores and PKC activators, such as phorbol esters, can reproduce all the downstream biological responses normally promoted by

PLC stimulation, including exocytosis of secretory granules and NADPH-oxidase activation (Di Virgilio et al., 1984, Robinson et al., 1984).

PKC activation, via DAG, elicits a variety of cellular responses by phosphorylating target proteins on serine or threonine residues. With the advent of PKC activators, such as PMA and PKC inhibitors, such as staurosporine (Nakadate et al., 1988) and Ro-31-8220 (Keller & Niggli, 1993), studies investigating the role of PKC in the regulation of apoptosis are now possible. Although some physiological substrates of this enzyme have been identified, the functions of many of these proteins in the cell and the role phosphorylation plays in their activity is unknown.

1.6.2 Protein tyrosine kinases

Protein tyrosine kinase receptors, which mediate the biological functions of many peptide growth factors and cytokines have now been characterised (Ullrich & Schlessinger, 1990). All tyrosine kinase receptors are composed of three major domains; an extracellular domain connected via a single membrane-spanning domain to a cytoplasmic domain. Upon ligand binding, the extracellular domain transmits the biological signal to the cytoplasmic domain, whose role is to convey the biological signal to intracellular target proteins. In addition to the catalytic protein tyrosine kinase, the cytoplasmic domain also contains distinct regulatory sequences with tyrosine, serine and threonine phosphorylation sites. Ligand binding to the extracellular domain of receptor tyrosine kinases is followed by receptor dimerization, stimulation of protein tyrosine kinase activity and autophosphorylation. Autophosphorylation provides an important regulatory step which determines the selectivity of signalling pathways recruited by growth factor receptors.

Receptor tyrosine phosphorylation promotes interaction of the receptor with a number of target proteins, including phospholipase C-γ (PLC-γ) (Ronnstrand et al., 1992), growth-factor-receptor binding protein 2 (Grb2) (Arvidsson et al., 1994) and has been shown to activate several serine/threonine kinases such as the MAP kinases (Chao, 1992).

1.6.2.1 SH2-containing proteins

The tyrosine-phosphorylated regions in growth factor receptors function as high affinity binding sites for cellular proteins such as PLC (Burgess et al., 1990), and the association between these receptors and proteins is mediated by a conserved region known as src homology 2 domains (SH2) (Koch et al., 1991, Parsons & Parsons, 1997). SH2 domains represent recognition motifs for specific tyrosine-phosphorylated peptide sequences, and are usually accompanied by a SH3 domain, the function of which is still poorly understood.

Proteins containing SH2 and SH3 domains can be divided into two groups (Mayer & Baltimore, 1993). Type I defines proteins that contain, in addition to the SH2 (and usually also SH3) domains, various catalytic activities, while type II contains only SH2 and SH3 domains. Thus, PLC-γ represents an example of a type I protein (Kim et al., 1991) and Grb2 represents an example of a type II protein (Lowenstein et al., 1992, Ren et al., 1993). Type II SH2-containing proteins therefore merely serve as adapter molecules to couple the activated receptor to other intermediates.

1.6.2.2 Cytokine-mediated receptor signalling with no intrinsic kinase activity

The IL-3, IL-5 and GM-CSF receptors share a common signal transducer that possesses no intrinsic kinase domain. These cytokine receptors are heterodimers of α- and β-subunits (Lopez et al., 1992), with the α-subunit being specific to each and the β-subunit common to all three receptors. Despite the absence of kinase domains in their receptors, these cytokines induce tyrosine phosphorylation of cellular substrate proteins and of their receptors, which has led to the assumption that a protein tyrosine kinase physically associates with their receptors and becomes activated following ligand binding (Ihle et al., 1994). It has now been reported that upon ligand binding these cytokines activate JAK2, which is a member of the JAK family of protein kinases (Silvennoinen et al., 1993, Briscoe et al., 1994, Karnitz & Abraham, 1995). Activation of JAKs by cytokines leads to JAK tyrosine phosphorylation, increased catalytic activity and the ability to associate with cytokine receptors. Structurally, JAKs possess two kinase domains, however they are unique among other cytoplasmic tyrosine kinases as they lack SH2 and SH3 domains (Pawson & Gish, 1992).

JAKs phosphorylate a group of nuclear proteins called signal transducers and activators of transcription (STAT proteins) and as their name suggests, couple ligand binding and activation of gene expression (Briscoe et al., 1994, Ihle et al., 1994, Karnitz & Abraham, 1995). The STAT family of proteins is characterised by the presence of a carboxy-terminal SH3 domain followed by a SH2 domain, and have been shown to be activated in association with JAKs, by for example, IL-5 in eosinophils (Pazdrak et al., 1995, Van der Bruggen et al., 1995).

1.6.3 Mitogen-activated protein kinases

The MAP kinase (mitogen-activated protein kinase) pathway represents a signal transduction cascade that is highly conserved in evolutionary distinct organisms and has been demonstrated in a variety of cells of mammalian, amphibian and yeast ancestries (Pelech, 1993). As illustrated in figure 1.4, the 'MAP kinases' constitute a superfamily of proteins that includes the ERKs (extracellular-regulated kinases), JNKs (Jun amino-

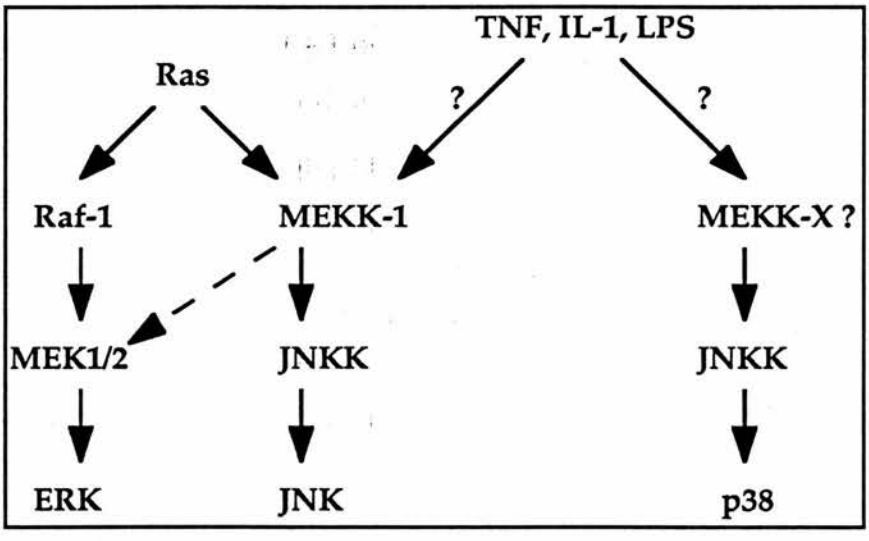

Figure 1.4. A schematic diagram illustrating the organisation of the three known mammalian MAP kinase cascades, leading to activation of ERK, JNK and p38. Abbreviations: MEK, MAP kinase kinase; MEKK-1, MEK kinase; MEKK-X, MEKK-like enzyme. (Modified diagram taken from Lin et al., 1995.)

terminal kinases) and p38 MAP kinases. Growth factors and mitogens preferentially activate ERK1 and ERK2, whereas inflammatory cytokines and various forms of stress preferentially activate JNK and/or p38 (Lin et al., 1995). With the advent of selective MAPK pathway inhibitors such as

PD 098059, which inhibits the MAPK/ERK cascade (Alessi et al., 1995, Dudley et al., 1995) and SB 203580, which inhibits the p38 MAPK cascade (Lee et al., 1994, Cuenda et al., 1995), it has become possible to investigate the influences of MAPKs on the regulation of apoptosis; discussed further in Chapter 5.

Several cytoplasmic signal transduction pathways involving sequential protein kinase reactions use the MAPK/ERK cascade. It is activated by phosphorylation on both tyrosine and threonine, a reaction which is in turn catalysed by a specific threonine/tyrosine directed kinase, MEK (MAPK/ERK kinase). MEK (MEK1/2) is phosphorylated and activated by the serine/threonine protein kinase, Raf. Recent evidence suggests the existence of several pathways to activation of MEK, including MEK kinase (MEKK) (Kyriakis et al., 1992, Lange-Carter et al., 1993) and Mos (Posada et al., 1993). However, the best delineated pathway indicates the involvement of Ras and Raf which phosphorylate MEK in response to tyrosine-linked receptors, such as FMLP receptors on neutrophils (Worthen et al., 1994). Regulation of MAPK/ERK involves an array of signals that ultimately converge on Raf.

Historically, this type of pathway has been associated with growth factor receptors which display intrinsic tyrosine kinase activity (Fantl et al., 1993), however a number of G-protein-coupled receptors have now also been shown to activate the MAP kinase cascade (Kahan et al., 1992). Receptors such as those for β-adrenergic agonists can activate the MAP kinase pathway and are coupled to the G proteins, Gi and Gq. Subsequent activation of αi, αq and βγ-complexes can regulate effectors that ultimately stimulate Raf activity, as illustrated in figure 1.5. The GTP-

bound αq subunit and the βγ-complexes can regulate phospholipase C-β (PLC-β) activity, leading to the activation of PKC. PKC directly phosphorylates Raf, however there are conflicting reports as to whether this results in Raf activation (Kolch et al., 1993, Macdonald et al., 1993). Interestingly, PKC-independent mechanisms are also able to stimulate the MAPK/ERK cascade, through the activation of Ras by heterotrimeric G proteins. Gi can activate the MAPK pathway by regulating GTP loading of Ras, however the precise steps involved are unknown. Such agents as thrombin and the mitogenic phospholipid lysophosphatidic acid (LPA) have been shown to activate the MAPK/ERK pathway (Kahan et al., 1992, Hordijk et al., 1994) by a mechanism independent of changes in PKC activation. Tyrosine kinases also activate Raf and the MAPK/ERK pathway. One such example is the epidermal growth factor (EGF)-receptor pathway, which involves the SH2 and SH3 domains of an adapter protein, Grb2, and the Ras guanine-nucleotide exchange factor, Sos. Growth factor binding results in receptor autophosphorylation and subsequent association between the Grb2 SH2 domain and a specific phosphotyrosine-containing sequence in the EGF-receptor (Egan et al., 1993, Buday & Downward, 1993). Sos, interacts with this receptor at the cytoplasmic surface of the plasma membrane, via the Grb2 SH3 domain and through formation of the Grb2-Sos complex, catalyses dissociation of GDP from Ras, allowing GTP loading and activation. The active Ras-GTP complex interacts directly with Raf (Van Aelst et al., 1993, Vojtek et al., 1993, Warne et al., 1993, Zhang et al., 1993) thereby recruiting Raf to the plasma membrane where it is activated by currently unidentified signals and by an as yet unidentified mechanism (Cai et al., 1993, Leevers et al., 1994, Stokoe et al., 1994). The presence of Raf as a major convergence point for different signals allows the integration of G protein and tyrosine

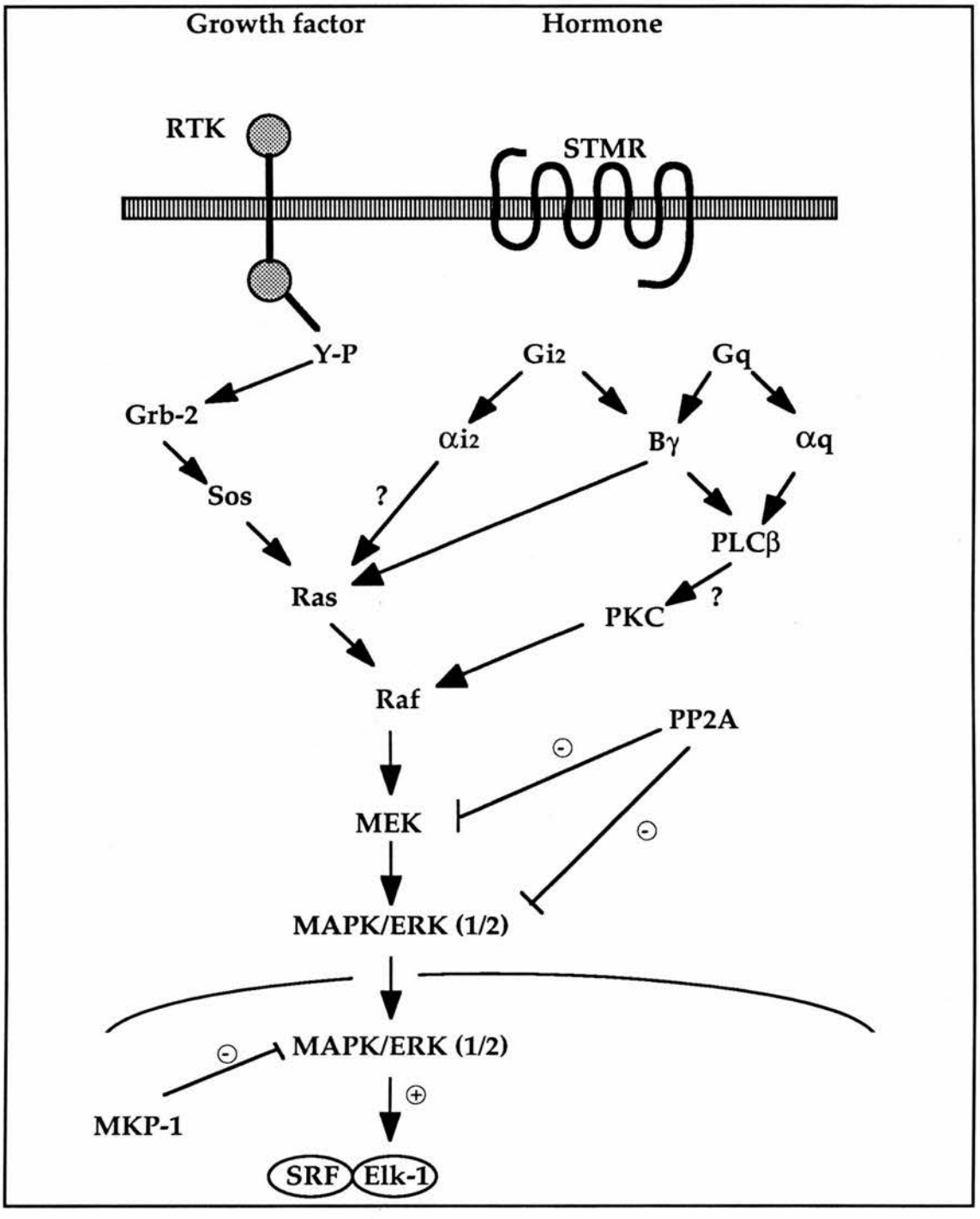

Figure 1.5. A simplified schematic diagram illustrating the MAP kinase (p42/p44) signalling cascade.
Autophosphorylation of tyrosine kinase encoded growth factor receptors and activation of STMR (receptors with seven transmembrane helices) coupled to heterotrimeric G proteins leads to the activation of Ras, and the ultimate translocation of ERK1/2 to the nucleus. Nucleus localised ERK phosphorylates and activates SRF/Elk-1 transcription complex. These cascades are inhibited by phosphatases PP2A and MKP-1. (Modified diagram from Blumer & Johnson, 1994.)

kinase activation of the MAPK/ERK cascade. Raf then phosphorylates and activates MEK (Dent et al., 1992, Howe et al., 1992, Kyriakis et al., 1992), which in turn phosphorylates and activates $p42^{MAPK}$ (ERK2) and $p44^{MAPK}$ (ERK1). The MAPKs/ERKs then phosphorylate and regulate a diverse group of proteins including cytoplasmic protein kinases (such as p90 ribosomal S6 kinase [Rsk]), phospholipases (such as cytosolic phospholipase A_2) and nuclear transcription factors (such as Elk-1) (Blenis, 1993). ERKs translocate to the nucleus and phosphorylate Elk-1 (a TCF, or ternary complex factor) on five residues near its carboxyl terminus (Gille et al., 1995). Elk-1 then associates with SRF (serum response factor), forming a ternary complex which binds the serum response element in the c-Fos promoter. Phosphorylation of Elk-1 leads to increased transcription of *c-Fos* mRNA, which is thought to result from increased transcriptional activation strength of the TCF/SRF complex (Treisman, 1994). In this way, MAPK/ERK transmits information to the nucleus, resulting in the regulation of genes involved in growth control.

As illustrated in figure 1.5, several of the components of the MAPK/ERK pathway are subject to regulation by protein phosphatases. The protein phosphatases, PP1 and PP2A, have been shown to dephosphorylate Raf *in vitro*, although the physiological protein phosphatase for Raf is unknown. Moreover, the activating phosphoserines in MEK and the activating phosphothreonines in ERK1/2 can be dephosphorylated *in vitro* by PP2A (Hunter, 1995). Furthermore, ERK1/2 can also be inactivated *in vivo* by the dual specificity protein phosphatases, MKP-1 (CL 100, 3CH 134) and PAC-1 (Ward et al., 1994), which have been reported to dephosphorylate both phosphothreonine and phosphotyrosine

residues. Interestingly these protein phosphatases have been reported to exhibit considerable specificity for individual MAPKs (Keyse, 1995).

1.6.4 Protein phosphatases

Protein phosphatases are a diverse group of proteins that have been classified based on various biochemical parameters. The serine/threonine-specific protein phosphatases were initially divided into two classes: type-1 phosphatases (PP1) are inhibited by nanomolar concentrations of the thermostable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2) and preferentially dephosphorylate the β-subunit of phosphorylase kinase, whereas type-2 phosphatases are insensitive to the inhibitors and preferentially dephosphorylate the α-subunit of phosphorylase kinase (Wera & Hemmings, 1995). The type-2 phosphatases comprise three enzymes (PP2A, PP2B and PP2C) that can be distinguished by their requirement for cations. PP2A, like PP1, does not have an absolute requirement for bivalent cations, whereas PP2B (or calcineurin) and PP2C are Ca^{2+}/calmodulin- and Mg^{2+}-dependent, respectively (Ingebritsen & Cohen, 1983, Cohen, 1989).

The precise regulation of protein phosphatases indicates their central role in cellular regulation, and abnormal inhibition of these enzymes leads to disorders of cellular functions. The discovery of many cell permeable phosphatase inhibitors, such as okadaic acid and calyculin A, has revolutionised the study of the function of protein phosphatases in cellular regulation (Holmes & Boland, 1993). Okadaic acid is a complex fatty acid produced by marine dinoflagellates which accumulates in the digestive glands of shellfish and marine sponges such as *Halichondria okadii*. It is the major cause of diarrhetic shellfish poisoning (Haystead et

al., 1989) and a potent tumour promoter (Suganuma et al., 1988). It is a potent inhibitor of PP2A and PP1; preferentially inhibiting PP2A at lower concentrations and also, at higher concentrations, PP1. Calyculin A, which is also isolated from sea sponges although is structurally unrelated to okadaic acid, inhibits both PP1 and PP2A at nanomolar concentrations with equal potency (Ishihara et al., 1989).

The native structure of PP1 is a 1:1 complex between the catalytic and a number of different regulatory subunits (Hubbard & Cohen, 1993). These regulatory subunits have an important role in targeting the catalytic subunit towards specific subcellular locations and increasing activity towards particular substrates (Hubbard & Cohen, 1993). PP2A, however, exists as several trimeric holoenzyme forms. The core of these structures consists of a 36 kDa catalytic subunit (PP2AC), homologous to that of PP1 (Berndt et al., 1987), complexed with a regulatory subunit of 65 kDa (PR65 or A subunit). This core dimer associates with variable regulatory subunits of 55 kDa (PR55 or B subunit), 54 kDa (B' subunit), 72 kDa (PR72), 74 kDa (B'' subunit) or 130 kDa (PR 130) (Mumby & Walter, 1993, Mayer-Jaekel & Hemmings, 1994) and several isoforms of each subunit exist (Axton et al., 1990). Again, as for PP1, the regulatory subunits have an important role influencing the substrate specificity of the catalytic subunit (Agostinis et al., 1992, Cegielska et al., 1994).

1.6.5 Protein kinase A (PKA)

Formation of cAMP results from a complex cascade of interactions, initiated by the actions of agonists at a variety of receptors, such as β-adrenoceptors and certain PG receptors. The receptors are coupled via intermediary GTP-binding regulatory proteins to the membrane-

associated enzyme, adenylyl cyclase, which exists as a family of isoenzymes (Tang & Gilman, 1992). Agonist-stimulated activation of Gs (a stimulatory guanine nucleotide-binding regulatory protein) leads to subsequent activation of adenylyl cyclase which catalyses the conversion of ATP to cAMP (Levitzki, 1988). The major effector system of cAMP is PKA (or cAMP-dependent protein kinase), which is a multisubstrate enzyme existing as two major types, I and II and is composed of regulatory (RI, RII) and catalytic (C) subunits. A schematic diagram of the cAMP/PKA signalling cascade is featured in Chapter 6. RI, RII and C exist in subforms and several groups have now highlighted the diversity of PKA and its subunit composition (McKnight, 1991, Døskeland et al., 1993). The inactive PKA holoenzyme consists of two regulatory (R) and two catalytic (C) subunits. Dissociation of this tetrameric complex occurs upon binding of two cAMP molecules to each of the two regulatory subunits, allowing the free activated catalytic subunits to phosphorylate specific substrate proteins and thereby alter their activities (Whitehouse & Abayasekara, 1994). As explained in Chapter 6, the downstream effects of cAMP on gene transcription are thought to be predominantly mediated through PKA phosphorylation of the transcription factor CREB (Gonzalez & Montminy, 1989). Studies featured in this thesis, investigating the role of PKA in the regulation of granulocyte apoptosis, have in part relied upon the use of H-89, for which it has previously been demonstrated is a selective inhibitor of PKA *in vitro* (Chijiwa et al., 1990).

1.7 Aims/Scope of thesis

The aim of this thesis is to determine the effects and mechanisms of action of glucocorticoids on eosinophil and neutrophil apoptosis and to examine the effects of these agents on macrophage-mediated phagocytosis of apoptotic granulocytes. The results section of this thesis is divided into five chapters. The first results chapter (Chapter 3) describes the effect of glucocorticoids on eosinophilic and neutrophilic apoptosis. The second results chapter (Chapter 4) describes the role of calcium and protein kinase C in basal and glucocorticoid-mediated granulocyte apoptosis. The third results chapter (Chapter 5) details the role of MAPK/ERK and protein phosphatases in basal and glucocorticoid-mediated granulocyte apoptosis. The involvement of PKA (cAMP-dependent protein kinase) in basal and glucocorticoid-mediated myeloid cell apoptosis is detailed in the fourth results chapter (Chapter 6). The final results chapter (Chapter 7) describes the effect of glucocorticoids on macrophage recognition of aged granulocytes.

Chapter 2

Materials and Methods

2.1 Materials

The following reagents were obtained from Gibco Life Technologies (Paisley, Scotland, UK): Hanks balanced salt solutions (HBSS), pH 7.4 and HBSS with 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}, pH 7.4 (HBSS with divalent cations) and Iscove's Dulbecco's modified Eagles medium, without supplement with L-glutamine (Iscove's DMEM) and culture supplements Penicillin (50 U/ml)/Streptomycin (50 U/ml).

The following reagents were obtained from the Sigma Chemical Company (Poole, Dorset, UK): bacitracin (dissolved in dH_20 as required), benzamidine (dissolved in dH_20 as required), 8-bromoadenosine 5'-monophosphate (dissolved in Iscove's DMEM at 20 mM and stored at -20°C), A23187 (dissolved in DMSO at 10 mM and stored at 4°C), cycloheximide (dissolved in DMSO at 10 mM and stored at -20°C), dextran-500 (dissolved in sterile 0.9% saline (6% w/v) and stored at 4°C), dimethoxybenzidine (o-DMB) (dissolved in sterile H_20 at 10 mg/ml and stored at -20°C), dimethyl sulfoxide (DMSO), (dl) dithiothreitol (DTT) (dissolved in dH_20 at 2 mM as required), N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl-cAMP) (dissolved in Iscove's DMEM at 20 mM and stored at -20°C), forskolin (dissolved in DMSO at 0.2 mM and stored at room temperature), fura-2 acetoxymethyl ester (fura-2/AM) (dissolved in DMSO at 1 mM and stored at -20°C), hydrocortisone (cortisol) (dissolved in Iscove's DMEM at 10 mM and stored at -20°C), 3-isobutyl-1-methylxanthine (IBMX) (dissolved in DMSO at 10 mM and

stored at -20°C), kemptide (dissolved in dH_20 at 2 mM and stored at -20°C), lipopolysaccharide (LPS; E coli 0127:B8) (dissolved in Iscove's DMEM at 10 mg/ml and stored at -20°C), phenylmethylsulfonylfluoride (PMSF) (dissolved in methanol as required), progesterone (dissolved in Iscove's DMEM at 10 mM and stored at -20°C), protein kinase inhibitor (IP_{20}) (dissolved in dH_20 at 10 μM and stored at -20°C), soybean trypsin inhibitor (dissolved in dH_20 as required), staurosporine (dissolved in DMSO at 1 mM and stored at 4°C) and thapsigargin (dissolved in DMSO at 10 mM and stored at -20°C).

The following reagents were obtained from Baxter Healthcare Ltd., (Baillieston, Glasgow, Scotland, UK): Diff-Quick stain [Solution I (Eosin G in phosphate buffer, pH 6.0); Solution II (Thiazine blue in phosphate buffer, pH 6.0)].

The following reagents were obtained from Calbiochem-Novabiochem Ltd., (Beeston, Nottingham, UK): 1,2-bis-(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) (dissolved in DMSO at 10 mM and stored at -20°C), calyculin A (dissolved in ethanol at 1 mM and stored at -20°C), N-[2-bromocinnamyl(amino)ethyl]-5-isoquinoline sulfonamide, HCl (H-89, dihydrochloride) (dissolved in ethanol at 5 mM and stored at -20°C), okadaic acid (dissolved in ethanol at 1 mM and stored at -20°C) and PD 098059 (dissolved in DMSO at 20 mM and stored at -20°C).

The following reagents were obtained from Cascade Biochem Ltd., (Reading, Berkshire, UK): prostaglandin D_2 (PGD_2) (dissolved in ethanol

at 2.84 mM and stored at -20°C) and 11-deoxy prostaglandin E_1 (PGE_1) (dissolved in ethanol at 2.95 mM and stored at -20°C).

The following reagents were obtained from Genzyme Diagnostics (Kent, UK): recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (dissolved in PBS without divalent cations at 1000 Units/ml and stored at -70°C) and monoclonal mouse anti-human GM-CSF (dissolved in Iscove's DMEM at a stock protein concentration of 1 mg/ml and stored at 4°C).

The following reagents were gifts from Glaxo-Welcome Group Research (UK): budesonide (dissolved in ethanol at 22 mM and stored at 4°C) and BW A868C (dissolved in ethanol at 2.18 mM and stored at -20°C).

The following reagents were obtained from Schering Health Care (West Sussex, UK): rolipram (dissolved in DMSO at 10 mM and stored at -20°C) and ZK 118.182 (dissolved in Iscove's DMEM as required and stored at -20°).

Dexamethasone was obtained from David Bull Laboratories (Warwick, UK) and stored at 4°C at a stock concentration of 8.3 mM. Dynabeads M-450 sheep anti-mouse IgG were obtained from Dynal (UK) Ltd., (Wirral, UK) and are supplied as a suspension containing $4x10^8$ beads/ml in phosphate buffered saline (PBS) pH 7.4 with 0.1% human serum albumin (HSA) and 0.2% sodium azide (NaN_3). Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin (Ig) were obtained from Dako Corporation (Buckinghamshire, UK). [γ-^{32}P]ATP was obtained from Amersham (Buckinghamshire, UK).

Interleukin 5 (IL-5) was obtained from R&D Systems Europe Ltd (Abingdon, UK) and was dissolved in PBS without divalent cations, containing 0.1% HSA at 2 μg/ml and stored at -70°C. Percoll and Q-sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Ro-31-8220 was obtained from Roche products Ltd (Welwyn Garden City, Herts, UK) and was dissolved in DMSO at 30 mM and stored at 4°C. RU38486 was kindly supplied by Roussel-UCLAF Center for Research (Romainville, France) and was dissolved, by extensive sonication, in PBS without divalent cations, containing 0.01% HSA at 10 mM and stored at -20°C . Sterile 3.8% sodium citrate was from Phoenix Pharmaceuticals (Gloucestershire, UK). The hybridoma clone 3G8 (CD16) was a generous gift from Dr. J. Unkeless, Mount Sinai Medical School, N.Y.

All other chemicals were of molecular, reagent or cell culture grade and were obtained from BDH (Leicestershire, UK).

2.2 Methods

2.2.1 Cell Purification

Human neutrophils and eosinophils were purified according to the methods previously described by Savill et al., (1989a) and Stern et al., (1992) respectively. Any donors with a history of atopy were asymptomatic at the time of the study and none of the donors were on glucocorticoid treatment. Cell isolation was performed under sterile conditions using LPS-free reagents and plastic ware (Falcon, Oxford, UK). Freshly drawn venous blood was collected and anticoagulated by addition to 50 ml polypropylene tubes, each containing 4 ml of sterile 3.8% sodium citrate solution (36 ml of blood was added to 4 ml citrate to make a total volume of 40 ml). The citrate and blood were mixed gently by inverting the tubes several times (in order to maintain the cells in a state of low activation) and then centrifuged (370g, 20 min). The upper layer, platelet-rich plasma (PRP), was aspirated taking care not to disturb the lower leukocyte/erythrocyte pellet and centrifuged (2400g, 20 min) to obtain platelet-poor plasma (PPP) or used to prepare autologous serum. The erythrocyte/leukocyte layer was then sedimented by adding 5 ml of 6% dextran (T500) [2.5 ml dextran per 10 ml cell pellet], and the volume made up to 50 ml with sterile 0.9% saline. The efficiency of dextran sedimentation is dependent upon temperature, thus 30 min prior to sedimentation the dextran and saline were warmed up to 37°C in a water bath. The cells were then allowed to sediment at room temperature for 30 min.

Autologous serum preparation. To a 15 ml glass tube, 10 ml of PRP was added followed by 220 µl of $CaCl_2$ at a final concentration of 22 mM

(containing 10 mmoles Ca^{2+} ions and 20 mmoles Cl^- ions in each 10 ml ampoule). This was left for 45 min, allowing recalcification, at 37°C to form a clot.

Platelet poor plasma (PPP) preparation. The remaining aspirated PRP was placed in a tube and centrifuged at 2400g for 20 min. The PPP was decanted off and the pelleted platelets were discarded.

2.2.1.1 Neutrophil preparation

The leukocyte-rich layer was aspirated from the sedimented red cells, centrifuged (235g, 6 min) and resuspended in 2.5 ml of 55% isotonic Percoll (9:1 vol./vol. Percoll:10 x PBS) in 1 x PBS without Ca^{2+}/Mg^{2+}. The cell fraction was then overlayed in 15 ml Falcon tubes onto 2.5 ml of 70% isotonic Percoll, previously overlayed on 2.5 ml of 81% isotonic Percoll. The gradients were centrifuged (720g, 20 min) and polymorphonuclear cells harvested from the 70%/81% Percoll interface. Mononuclear cells sedimented at the 55%/70% interface. Purified neutrophils were sequentially washed in PPP, HBSS without divalent cations and HBSS with divalent cations (235g, 6 min) and finally resuspended in Iscove's DMEM containing 5% (v/v) autologous serum. Density gradient centrifugation does not separate neutrophils from eosinophils or basophils, however harvested polymorphonuclear cells were generally >98% pure, with basophils rarely seen and preparations containing >5% eosinophils discarded.

2.2.1.2 Eosinophil preparation

Eosinophils (>98% pure on Diff-Quick stained cytopreparations) were isolated using a two stage procedure based on a modification of a

previously described method involving a combination of dextran sedimentation and centrifugation through six isotonic discontinuous plasma-Percoll gradients to produce cell fractions containing neutrophils and eosinophils (Haslett et al., 1985). This was followed by immunomagnetic separation of eosinophils from contaminating neutrophils using the murine anti-neutrophil Ab 3G8 (anti-CD16)-coated sheep anti-mouse IgG-Dynabeads, by a process known as 'negative selection' (Bach et al., 1990). Cells were prepared from peripheral blood, as described in section 2.2.1. The leukocyte-rich layer was aspirated from the sedimented red cells, centrifuged (530g, 6 min) and finally re-suspended in 2 ml autologous PPP. Percoll-plasma gradients (60-77% Percoll) were prepared by diluting stock Percoll (9:1 vol./vol. Percoll:10 x PBS) in 1 x PBS without divalent cations and overlaying 2 ml of each gradient in a 15 ml Falcon tube. The leukocyte suspension was finally overlayed and the gradients centrifuged (570g, 40 min) at 22°C, with the brake and accelerator off. Eosinophilic and neutrophilic granulocytes were harvested from the 74%/77% interface and washed sequentially in PPP (370g, 5 min), followed by HBSS without divalent cations (260g, 5 min), before suspension in HBSS without divalent cations containing 0.1% gelatin. The cells were then counted and the percentage of neutrophil contamination in each fraction was assessed either by flow cytometry analysis involving antibody detection of cells expressing surface CD16 or morphologically by examination of cytocentrifuge preparations.

The CD16-Dynabeads were prepared under sterile conditions by combining 3G8 cell supernatants with dynal M450 sheep anti-mouse dynabeads (10 ml supt:700 µl dynabeads), in a 15 ml falcon tube and the

mixture was then left to rotate (to ensure maximal coating of beads with antibody) at 4°C overnight. Coated beads were washed three times in HBSS without divalent cations containing 0.1% gelatin and the beads retrieved by stationary contact (5 min) using a magnet (Dynal Magnetic Particle Concentrator, MPC-1). Cells were mixed with washed 3G8-Dynabeads at a bead:neutrophil ratio of 3:2 on a rotary mixer at 4°C for 20 min, and the beads with attached neutrophils were removed magnetically by stationary contact (3 min) with a magnet. This procedure was repeated once. Purified eosinophils (>99% pure with neutrophils as the major contaminant) were washed twice in 8 ml of HBSS without divalent cations containing 0.1% gelatin by centrifugation (235g, 5 min) and finally resuspended in Iscove's DMEM containing 5% (v/v) autologous serum.

Alternatively, eosinophils were isolated using a two step procedure involving separation of dextran buoyant leukocytes into fractions and centrifugation through three plasma-Percoll gradients (as described in section 2.2.1.1), followed by extraction of the contaminating neutrophils using the murine anti-neutrophil Ab 3G8 (anti-CD16)-coated sheep anti-mouse IgG-Dynabeads (as described in section 2.2.1.2). Purified eosinophils (>99% pure with neutrophils as the major contaminant) were washed twice in 8 ml of HBSS without divalent cations containing 0.1% gelatin by centrifugation (235g, 5 min) and finally resuspended in Iscove's DMEM containing 5% (v/v) autologous serum.

2.2.1.3 Mononuclear cell preparation

Mononuclear cells (MNC) were isolated from peripheral blood and aspirated from the 55%/70% interface, which was prepared as described in

section 2.2.1.1. MNCs were sequentially washed in HBSS without divalent cations, followed by HBSS with divalent cations (235g, 6 min) and finally resuspended in Iscove's DMEM alone for production of monocyte-derived macrophages (see section 2.2.2.3).

2.2.2 Cell culture

2.2.2.1 Neutrophils

Purified neutrophils were routinely suspended at a density of $2x10^6$/ml in Iscove's DMEM supplemented with 5% autologous serum and 50 U/ml penicillin and 50 U/ml streptomycin. Neutrophils ($3x10^5$) were cultured in flat-bottomed 96 well Falcon flexiwell plates, in a final volume of 150 µl, at 37°C in a humidified 5% CO_2 atmosphere, for the time periods indicated. Reagents to be examined in this assay system were diluted to 10x the final concentration required in Iscove's DMEM.

2.2.2.2 Eosinophils

Purified eosinophils were routinely suspended at a density of $2x10^6$/ml in Iscove's DMEM supplemented with 5% autologous serum and 50 U/ml penicillin and 50 U/ml streptomycin. Eosinophils ($3x10^5$) were maintained in flat-bottom flexible wells, in a final volume of 150 µl, at 37°C in a 5% CO_2 atmosphere for the time periods indicated. Reagents to be examined in this assay system were diluted to 10x the final concentration required in Iscove's DMEM.

2.2.2.3 Macrophages

Mononuclear cells (MNC) were resuspended at $4x10^6$/ml in Iscove's DMEM and at a volume of 1 ml per well, were allowed to adhere to 24

well tissue culture plates for one hour at 37°C. The non-adherent cells (lymphocytes) were removed by washing each well three times, in warm HBSS with divalent cations. Monocytes were then cultured for six days in Iscove's DMEM containing 5% autologous serum, with fresh medium added on day 3, as previously described by Newman et al., (1982).

2.2.3 Assessment of apoptosis

Four different methods were employed to enable the assessment of apoptosis.

2.2.3.1 Assessment of apoptosis by morphological criteria

Cells were removed from culture at various times, as indicated in the *Results*, for assessment of morphology according to the method of Savill et al., (1989a). Cytocentrifuge preparations of cell samples in each experiment were prepared in a Shandon Cytospin II (Shandon, UK), fixed in methanol, and stained with Diff-Quick. Cells were examined under oil immersion light microscopy (x1250 magnification), and apoptotic cells were defined as those containing one or more darkly stained pyknotic nuclei (see Chapter 3; figures 3.2 and 3.3). For each condition examined, slides were prepared from duplicate or triplicate incubations and a total of at least 500 neutrophils were counted over five fields of view, with the observer blinded to the assay conditions.

2.2.3.1.1 Cell viability and recovery

Cell viability was determined as the ability to exclude trypan blue dye (10 µl of cells:40 µl of trypan blue). Cell recovery was determined by counting the number of granulocytes occupying 25 squares of the haemocytometer.

From this value the percentage of cells retrieved after the experiment, compared with the number of cells added to each well initially was estimated. Thus, at an initial cell concentration of $2x10^6$/ml, cell recovery was calculated using the following formula:

$$\text{Recovery} = \frac{\text{No. of cells in 25 squares}}{200}$$

2.2.3.2 Propidium iodide staining

Apoptosis in both neutrophils and eosinophils was also assessed by analysis of the staining characteristics of fixed/permeabilised cells exposed to the DNA-binding dye propidium iodide, using a modification of the method previously described by Nicoletti et al., (1991). Neutrophils ($2x10^6$/ml) were added to 96-well flexible assay plates, washed in cold PBS (235g, 1 min) and fixed for 10 min in ice-cold 70% ethanol. Fixed cells were then washed twice in 200 µl of cold PBS (235g, 6 min) and resuspended finally in 150 µl of PBS containing 1.3 mg/ml RNase (Sigma Chemical Co.) and 33 µg/ml of propidium iodide (Sigma Chemical Co.). The suspension was incubated in the dark at room temperature for 15 min before analysis on an EPICS Profile II (Coulter Electronics, Luton, UK). The proportion of cells that display a "hypodiploid" peak has been shown to correlate with apoptosis (Dransfield et al., 1994).

2.2.3.3 Assessment of neutrophil CD16 expression

Neutrophil apoptosis was assessed by antibody detection of cells with low levels of surface CD16 (FcγRIII). Antibody labelling of intact cells was performed as described previously by Dransfield et al., (1994). Neutrophil cell surface CD16 was labelled with mAb 3G8 and compared with a control mAb MOPC 21C (European Animal Cell Culture Collection,

Porton Down, UK). The detecting Ab was FITC-conjugated $F(ab')_2$ rabbit anti-mouse Ig (Dako Corp., High Wycombe, UK). Neutrophils ($2x10^6$/ml) were added to 96-well flexible assay plates and washed in Tris-buffered saline containing 0.2% BSA, 0.1% sodium azide (TBN). Cells were then incubated in 50 µl of saturating concentrations (20 µg/ml) of primary Ab (3G8 supernatant) for 30 min on ice, washed twice in TBN (235g, 1 min) and incubated in 30 µl of 1/25 dilution of secondary Ab, FITC-conjugated $F(ab')_2$ rabbit Ab to mouse Igs for 30 min on ice. Cells were washed twice in TBN (235g, 1 min) and analysed by flow cytometry on an EPICS Profile II (Coulter Electronics, Luton, UK).

2.2.4 Determination of cytosolic free calcium ion levels ($[Ca^{2+}]_i$)

2.2.4.1 Fluorometric measurements

$[Ca^{2+}]_i$ was assayed using a modification of the method described by Whyte et al., (1993b). Neutrophils were removed from culture, washed three times in HBSS without phenol red and divalent cations, before being resuspended at 10^7/ml in HBSS without divalent cations, for incubation with fura-2/AM (final concentration 2 µM) for 30 mins at 37°C. The cells were then washed twice to remove fura-2/AM and left in HBSS without divalent cations for a further 10 mins for optimal deesterification, before finally resuspending the neutrophils at $2x10^6$/ml in HBSS without phenol red but with divalent cations. The changes in fluorescence upon agonist addition were determined using a 'SPEX Fluoromax' fluorimeter, with dual wavelength excitation (340 and 380 nm), emission at 510 nm, fitted with a thermostated cuvette compartment and stirring attachment, to ensure complete mixing of reagents.

2.2.4.2 Calibration of the fluorescence

This was carried out prior to the experiment by measuring the maximal fluorescence (R_{max}) and the minimum fluorescence (R_{min}). R_{max} is therefore the ratio obtained in the presence of saturating $[Ca^{2+}]_i$ (after treatment with 50 μM digitonin). R_{min} is the ratio obtained in the absence of Ca^{2+} (addition of 25 mM EGTA).

$[Ca^{2+}]_i$ was calculated from the relationship $[Ca^{2+}]_i = Kd.(R-R_{min})/(R_{max}-R).\beta$: where $[Ca^{2+}]_i$ is the cytosolic calcium concentration, R is the ratio of fluorescence obtained at 340 and 380 nm in the cuvette before calibration, R_{max} is the fluorescence ratio under saturating $[Ca^{2+}]_i$, R_{min} is the fluorescence ratio in the absence of Ca^{2+}, Kd is the dissociation constant for fura-2/AM, taken as 224 nm at 37°C and β is the fluorescence ratio at 340 nm of cells in the absence and presence of Ca^{2+}.

2.2.5 Macrophage recognition assay

2.2.5.1 Monocyte-derived macrophage culture

Mononuclear cells (MNC) were cultured as described in section 2.2.2.3.

2.2.5.2 Phagocytic assay for apoptotic granulocytes

Recognition of aged neutrophils and eosinophils by macrophages matured from monocytes over 7 days, was assayed by a method previously described by Newman et al., (1982). Aged granulocytes were washed in Iscove's DMEM, suspended at $4x10^6$/ml in Iscove's, and 1 ml of suspension was added to each well of adherent macrophages cultured in 24-well plates. After 30 min incubation at 37°C in a 5% CO_2 atmosphere, the wells were vigorously washed with cold HBSS without

divalent cations, fixed with 2.5% glutaraldehyde in PBS without divalent cations, and then stained for peroxidase activity using hydrogen peroxide and dimethoxybenzidine as substrate. The interaction was quantified by counting, under light microscopy, the proportion of macrophages containing peroxidase positive cells in five randomly selected fields, with at least 500 macrophages being assayed per well.

2.2.5.3 Phagocytic assay for IgG-opsonised erythrocytes

Erythrocytes were obtained from the red blood cell pellet following dextran sedimentation, described in section 2.2.1, and washed in PBS without divalent cations (530g, 5 min). The cells were then re-suspended in 50 ml PBS without divalent cations and stored at 4°C overnight, for use on the day of the macrophage interaction assay.

Erythrocytes were centrifuged (530g, 5 min) and 200 µl of the pellet was re-suspended in 10 ml PBS without, in a 15 ml Falcon tube, and incubated at 37°C with rabbit IgG to human erythrocyte membrane (1:4000) for 1 hr. Opsonised erythrocytes were then washed twice in PBS without divalent cations (528g, 5 min), re-suspended in Iscove's DMEM at a final concentration of $4x10^6$/ml and incubated with macrophages on 24 well tissue culture plates, as described for apoptotic granulocytes in section 2.2.5.2. After 30 min, the wells were washed once in ice cold HBSS with divalent cations and non-phagocytosed erythrocytes were lysed by addition of one drop of dH_2O per well for approximately 10 s, after which time PBS without divalent cations was added to the wells. The cells were then fixed with glutaraldehyde and stained for peroxidase activity as described in section 2.2.5.2.

2.2.5.4 Discrimination between ingestion and binding of apoptotic neutrophils

The macrophage interaction was carried out as described in section 2.2.5.2, however instead of fixing, 150 µl of 0.25% trypsin, 0.02% EDTA was added to each well and incubated at 37°C for 10 min. The cells were then mixed with 20 µl of FCS and examined cytologically. The cytospins were allowed to dry before addition of 50 µl of 2% formaldehyde for 10 min, peroxidase activity staining for 30 min with hydrogen peroxide and dimethoxybenzidine and finally counter-staining with Diff-Quick for 5 s, for microscopic examination to confirm the presence of neutrophilic granulocytes within the macrophages.

2.2.6 Protein kinase A (PKA) studies

2.2.6.1 Chromatographic separation of A-kinase isoenzymes

Unless otherwise stated all chromatographic procedures were performed at 4 °C. For the separation, $200x10^6$ neutrophils were lysed for 15 min, by addition of 5 ml of ice-cold buffer A (10 mM HEPES, pH 7.2, 1 mM EDTA, 2 mM DTT, 1% (v/v) Triton X-100) containing 100 µM PMSF, 10 µg/ml soybean trypsin inhibitor, 20 µg/ml benzamidine and 100 µg/ml bacitracin. Cells were then centrifuged (31,000 g, 30 min) at 4 °C. The soluble enzyme (supernatant) was diluted in 5 ml of buffer B (buffer A minus triton) and then applied to a column (Pharmacia; 1x6 cm) of Q-sepharose, pre-equilibrated with buffer B. The column was then washed with a further 40 ml of the same buffer to remove unbound protein and A-kinase enzymes were eluted with a linear NaCl gradient running from 0 to 320 mM in a total volume of 60 ml. The flow rate was adjusted to 0.5 ml/min and 40x1 ml fractions were collected. Aliquots of each fraction were then assayed for A-kinase activity as described overleaf.

2.2.6.2 Measurement of PKA activity

Neutrophils ($2x10^6$/ml) were cultured in the absence and presence of various reagents illustrated in Chapter 6, *results* and at the appropriate time period were washed and lysed ($10x10^6$/ml), for 15 min, in buffer A. The resulting lysed solution of cells was used as the source of soluble enzyme.

Soluble A-kinase activity was measured using a modification of methods previously described by Witt & Roskoski, (1975) and Giembycz & Diamond, (1990). Assays were performed in triplicate at 30°C and initiated by the addition of 25 µl of the soluble extract to 75 µl of a reaction medium (pH 7.2) containing (final concentration): 20 mM HEPES, 10 mM magnesium acetate, 2 mg/ml BSA, 100 µM IBMX, 100 µM ATP supplemented with ~ 100 cpm/pmol [γ-^{32}P]ATP and 100 µM kemptide in the absence and presence of 10 µM cAMP. In the chromatographic separation of A-kinase isoenzymes, phosphotransferase activity was defined with IP_{20} (1 µM), a synthetic icosapeptide inhibitor of PKA (Cheng et al., 1986). Reactions were terminated after 30 min by pipetting 50 µl aliquots of the mixture onto 2x2 cm phosphocellulose paper squares (Whatman P81), which were left for 30 s before being immersed in 150 mM phosphoric acid. The paper squares were extensively washed in 4 litres of fresh phosphoric acid, immersed in industrial methylated spirits and allowed to dry. Bound radioactivity (representing phosphokemptide) was subsequently quantified by liquid scintillation counting. One unit of phosphotransferase activity was defined as the amount of PKA that catalysed the incorporation of 1 pmol of phosphate from ATP into kemptide/min/10^6 cells at 30°C.

2.2.7 Statistical analysis

All data are presented as mean ± SEM of the indicated number of experiments. Data were analysed by the students t-test and where appropriate by one way analysis of variance followed by Newman-Keuls procedure. P values less than 0.05 were considered significant.

Data are presented as the percentage of apoptotic cells from raw data counts, involving the proportion of cells exhibiting apoptotic morphology in five different fields of view, for each cytospin preparation (see *section 2.2.3.1*).

Chapter 3

Effect of glucocorticoids on the rate of eosinophil and neutrophil apoptosis

3.1 Introduction

Eosinophilic and neutrophilic granulocytes are key effector cells mediating host defence against inciting bacteria and parasites by a variety of mechanisms such as phagocytosis and secretion of cytotoxic and degradative enzymes. Inflammatory infiltrates of these granulocytes has been associated with the pathogenesis of many diseases, with allergic and asthmatic reations being characterised by a predominantly eosinophilic infiltrate (Wardlaw, 1996, Azzawi et al., 1992, Sur et al., 1993) and diseases such as rheumatoid arthritis having a prominent neutrophil involvement (Brown, 1988). Efficient resolution of inflammation therefore relies upon the existence of a fine balance between proficient tissue protective mechanisms and potentially detrimental effects of inflammation to the host.

Clinically, glucocorticoids have been used as potent anti-inflammatory agents, for the treatment of hypereosinophilia in a range of diseases. However, the mechanisms underlying the anti-inflammatory advantage conferred by glucocorticoids *in vivo* are poorly understood (Barnes, 1995b, Goulding & Guyre, 1993). Glucocorticoids readily penetrate the cell membrane and bind directly to cytosolic glucocorticoid receptors, which, when activated, exhibit DNA-binding ability and thereby interact with a wide range of nuclear targets, regulating both the positive and negative expression of specific proteins (Maroder et al., 1993). The removal of

eosinophils and neutrophils from an inflamed site is a pre-requisite for the resolution of inflammation to occur. Apoptosis provides an efficient mechanism for the clearance of unwanted cells, representing a tissue injury-limiting mechanism, promoting the resolution of inflammation. Granulocytes undergoing apoptosis are ingested, while still intact, by macrophages, (Stern et al., 1992, Savill et al., 1989a), which are involved in the specific recognition of apoptotic cells without inciting inflammatory sequelae (Meagher et al., 1992, Stern et al., 1996). Clearance of apoptotic granulocytes by macrophages may therefore be a central component of the resolution or limitation of inflammation, removing these cells before the onset of necrosis and thereby preventing disgorgement of their potentially histotoxic contents (Haslett et al., 1989).

Despite their close haemopoietic origins, tissue longevity of eosinophils and neutrophils, like granulocytopoeisis, is controlled by different external factors. It has now been ascertained that these observations may be explained by inhibition of the process of apoptosis, which precedes necrosis in cultured eosinophils and neutrophils (Brach et al., 1992, Lee et al., 1993). Thus, granulocyte apoptosis is an immutable process, being delayed in the presence of inflammatory mediators such as GM-CSF, complement factor C5a and LPS (Lee et al., 1993, Begley et al., 1986, Lopez et al., 1986, Brach et al., 1992, Takanaski et al., 1994, Cox et al., 1992). Eosinophils also respond specifically to IL-3 and IL-5 (which have no effect on neutrophils), and inclusion of IL-5 and IL-3 in eosinophil culture medium prolongs eosinophil lifespan by inhibiting apoptosis (Stern et al., 1992, Her et al., 1991, Yamaguchi et al., 1991, Rothenberg et al., 1988, Tai et al., 1991, Matsumoto et al., 1995). Thus, certain cytokine environments

favour the survival of eosinophils while neutrophil survival is unaffected, by virtue of cell-selective activation.

Many of the mechanisms that initiate and amplify inflammation are susceptible to inhibition by corticosteroids. Thus macrophage production of cytokines such as IL-1, is suppressed by corticosteroids (Kern et al., 1988), as are the eosinophil life-prolonging effects of GM-CSF (Her et al., 1991, Lamas et al., 1991) and IL-5 (Hallsworth et al., 1992). Dexamethasone is known to induce apoptosis in a variety of different cell types, yet direct effects of glucocorticoids upon the apoptotic programme of myeloid cells have remained obscure. The purpose of these studies was to investigate the direct effects of dexamethasone on the rate of eosinophil and neutrophil apoptosis in the absence of exogenous cytokines.

3.2 Results

3.2.1 Effect of dexamethasone on the rate of eosinophil and neutrophil apoptosis *in vitro*

Eosinophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM showed increased apoptosis in the presence of 1 μM dexamethasone (Figure 3.1*A*). Assessment of cell viability and recovery demonstrated that dexamethasone did not significantly alter either of these parameters (data not shown), excluding the possibility that the increased rates of apoptosis observed reflected either dexamethasone-induced adhesion of non-apoptotic cells, or a dexamethasone-induced switch between necrosis and apoptosis seen in a number of distinct cell lines, including HL-60 cells, treated with high concentrations of a variety of death-inducing agents (Lennon et al., 1991). The time point of 40 h was chosen to take advantage of the temporal separation of apoptosis from necrosis as described previously (Stern et al., 1992), allowing analysis of apoptotic cultures before the onset of necrosis some hours later. These results demonstrate a direct positive effect of dexamethasone on eosinophil apoptosis.

In contrast to the effects observed on eosinophils, neutrophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM showed decreased apoptosis in the presence of 1 μM dexamethasone (Figure 3.1*B*), without adversely affecting cell viability or recovery. The time period of 20 h was chosen because it preceded the onset of significant necrosis (Savill et al., 1989a). Thus, dexamethasone directly inhibits neutrophil apoptosis.

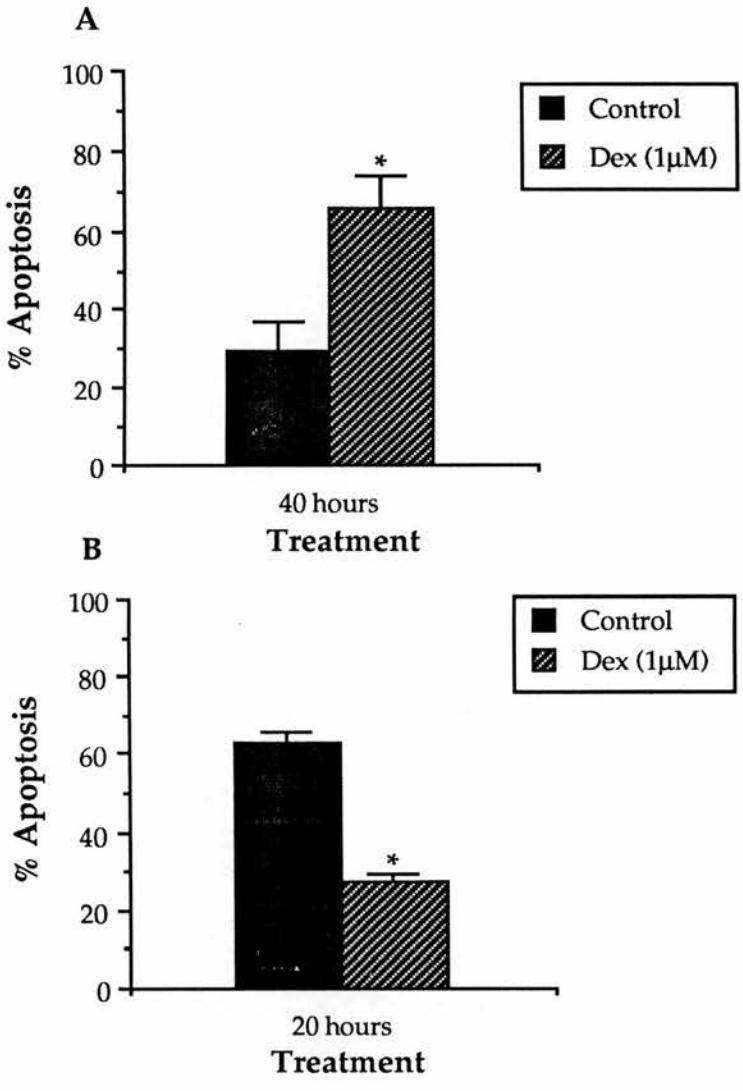

Figure 3.1 Effect of dexamethasone on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (1 μM). Eosinophils were harvested following 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 12 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 55 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

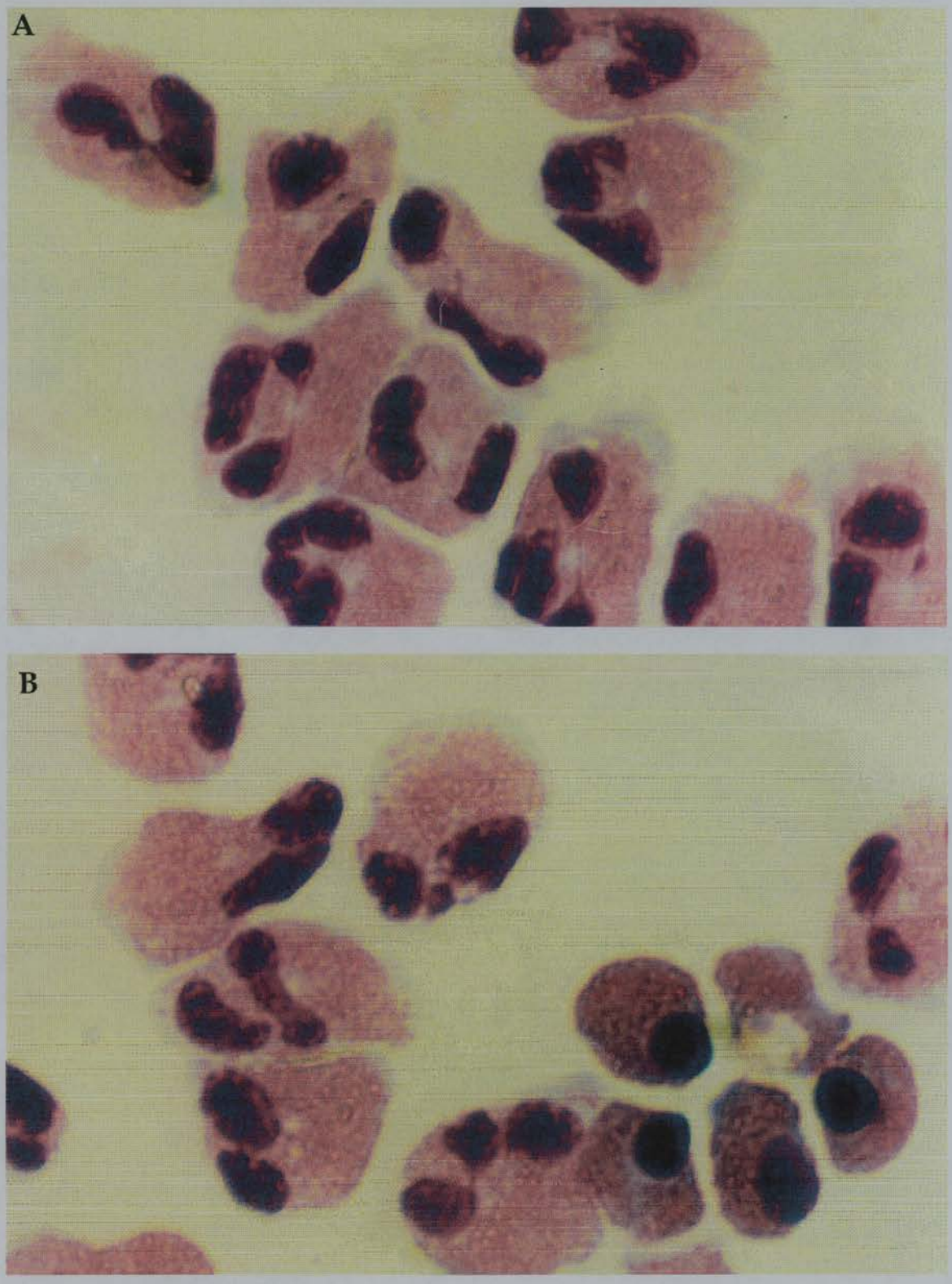

Figure 3.2 Effect of dexamethasone on eosinophil morphology.
Photomicrographs (x1250 magnification) of cytocentrifuge preparations of cultured human eosinophils isolated from the peripheral blood of a single donor and harvested following 40 h in culture. Cells were cultured alone *(A)* or in the presence of 1 μM dexamethasone *(B)*.

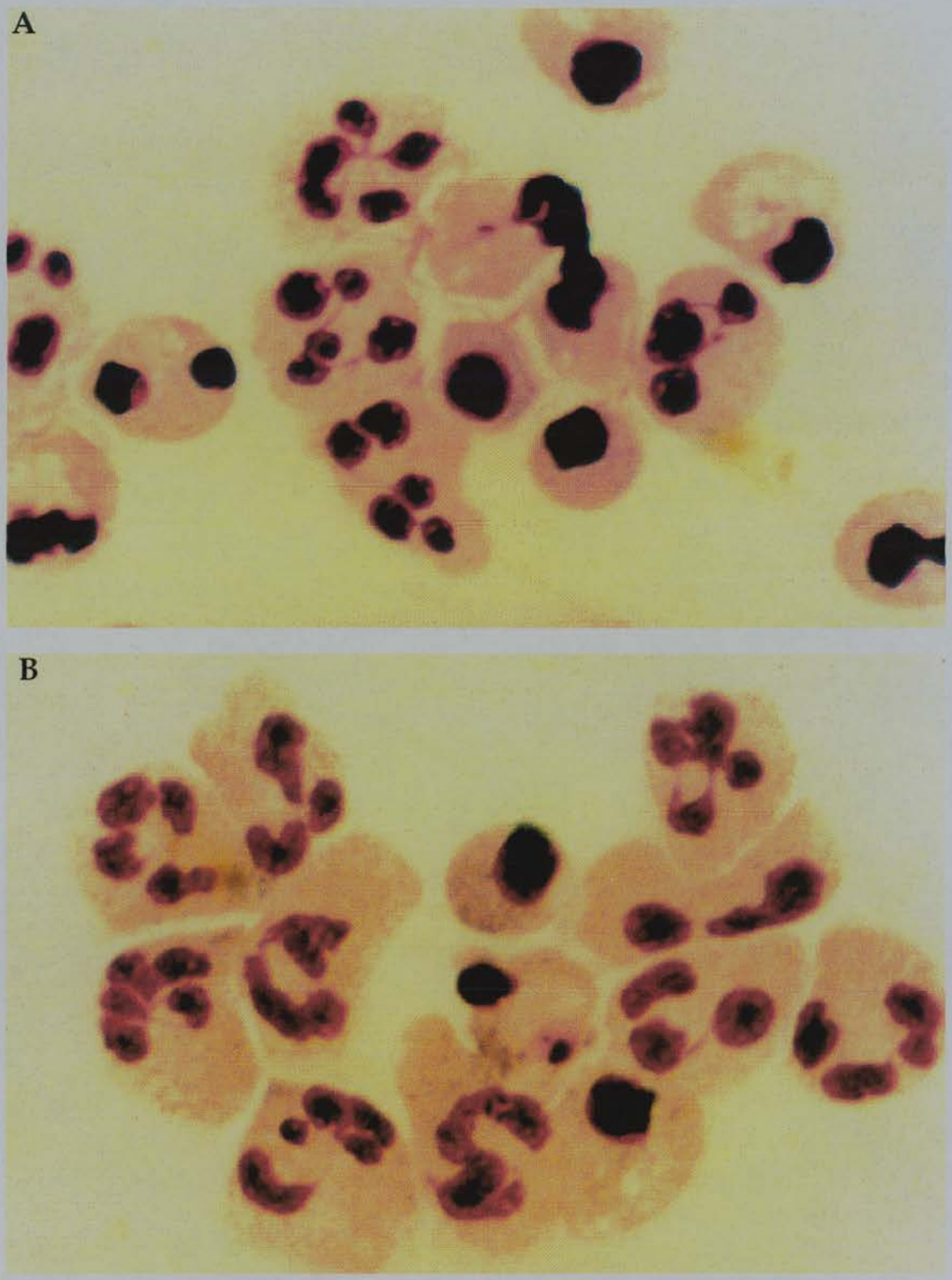

Figure 3.3 Effect of dexamethasone on neutrophil morphology.
Photomicrographs (x1250 magnification) of cytocentrifuge preparations of cultured human neutrophils isolated from the peripheral blood of a single donor and harvested following 20 h in culture. Cells were cultured alone *(A)* or in the presence of 1 μM dexamethasone *(B)*.

3.2.2 Assessment of dexamethasone-mediated eosinophil and neutrophil apoptosis

Eosinophils cultured for 40 h in the presence of dexamethasone (1 μM) showed light microscopic features characteristic of apoptosis (Wyllie et al., 1980), as shown in figure 3.2. Morphological changes included nuclear pyknosis or chromatin condensation, together with decreased cell size and cytoplasmic vacuolation. Numerous anucleate eosinophils or "ghosts" habitually appeared on the cytocentrifuge preparations, indicative of nuclear extrusion, which is another morphological feature associated with apoptosis. On light microscopy of 20 h aged neutrophils, a reduction in the number of apoptotic cells was seen in dexamethasone-treated cultures, by comparison with untreated controls (figure 3.3). Furthermore, parallel analysis of aged cell cultures by morphologic examination and staining with the DNA-binding dye propidium iodide showed the predicted reduction in the proportion of cells generating the hypodiploid peak, which corresponds with the apoptotic subpopulation (Nicoletti et al., 1991) in dexamethasone-treated cultures, by comparison with untreated controls (figure 3.4, *A and B*). Similarly, dexamethasone-treated eosinophil cultures showed increased proportions of apoptotic cells by analysis of propidium iodide staining characteristics (figure 3.4, *C and D*), in agreement with morphologic data. Figure 3.4 illustrates that the percentage of cells within the hypodiploid or apoptotic gate (gate B) was reduced by dexamethasone treatment of neutrophils from 69.1% (*A*; control) to 57.0% (*B*) and increased in eosinophils from 18.5% (*C*; control) to 29.8%(*D*). The percentages of apoptosis in parallel cultures were assessed morphologically as: *A*, 81.2%; *B*, 44.1%; *C*, 16.9%; and *D*, 27.9%. These data confirm the close correlation that exists between these assessment methods.

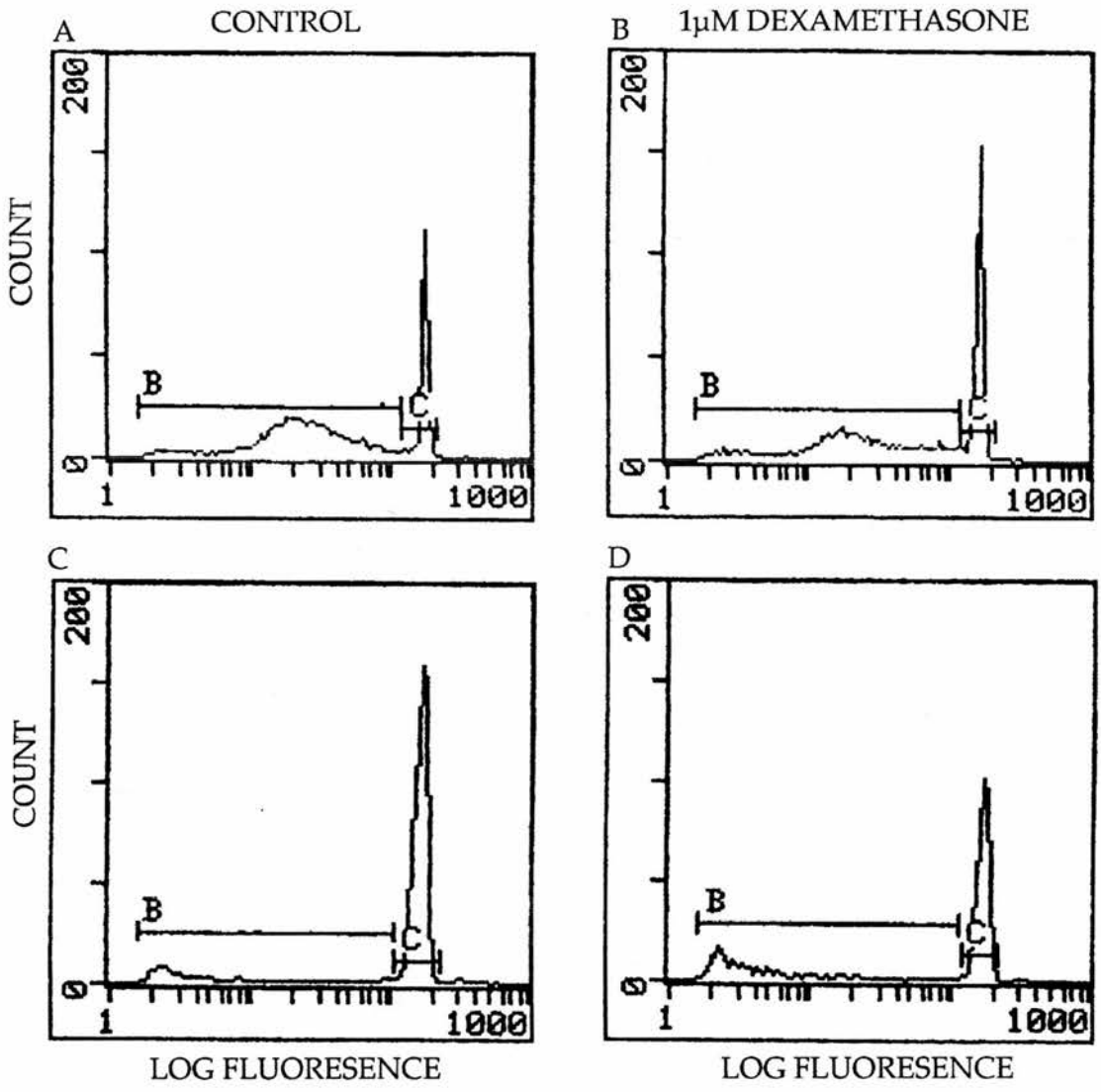

Figure 3.4 Effect of dexamethasone on neutrophils and eosinophils as assessed by propidium iodide staining.
Neutrophils *(A and B)* and eosinophils *(C and D)* that had been cultured under control conditions *(A and C)* or in the presence of 1 μM dexamethasone *(B and D)* for 20 h were stained with propidium iodide and analysed by flow cytometry. The results presented are from a representative experiment.

A reduction in cell surface expression of CD16 has been shown to be associated with apoptosis in neutrophils (Dransfield et al., 1994). In agreement with this, figure 3.5 shows that dexamethasone induced a reduction in the number of cells expressing low levels of CD16. The CD16 "low" peak in the control 20 h cells (figure 3.5*A*) represents apoptotic cells that have shed their surface CD16; the percentage of cells with "low" CD16 was markedly reduced when compared to cells that had been treated with dexamethasone (figure 3.5*B*), whereas the corresponding CD16 "high" peak was increased. Significantly, the percentage of apoptosis was reduced from 58.9% (A; control) to 13.7% (B; dexamethasone).

3.2.3 Time-course for the effect of dexamethasone on eosinophil and neutrophil apoptosis

Figure 3.6 examines the time-course of the effect of dexamethasone on the rate of eosinophil *(A)* and neutrophil *(B)* apoptosis. With longer incubation periods, there is a progressive increase in the constitutive rate of eosinophil apoptosis *in vitro* (Stern et al., 1992) and this time-dependent observation is further accelerated in the presence of dexamethasone (1 μM). Similarly, the constitutive rate of neutrophil apoptosis was shown to increase over time (Savill et al., 1989a), however in contrast to the eosinophil, in the presence of dexamethasone (1 μM) the rate of neutrophil apoptosis was delayed. Figure 3.6 also illustrates the slower constitutive rate of apoptosis over time in eosinophils, as compared to neutrophils.

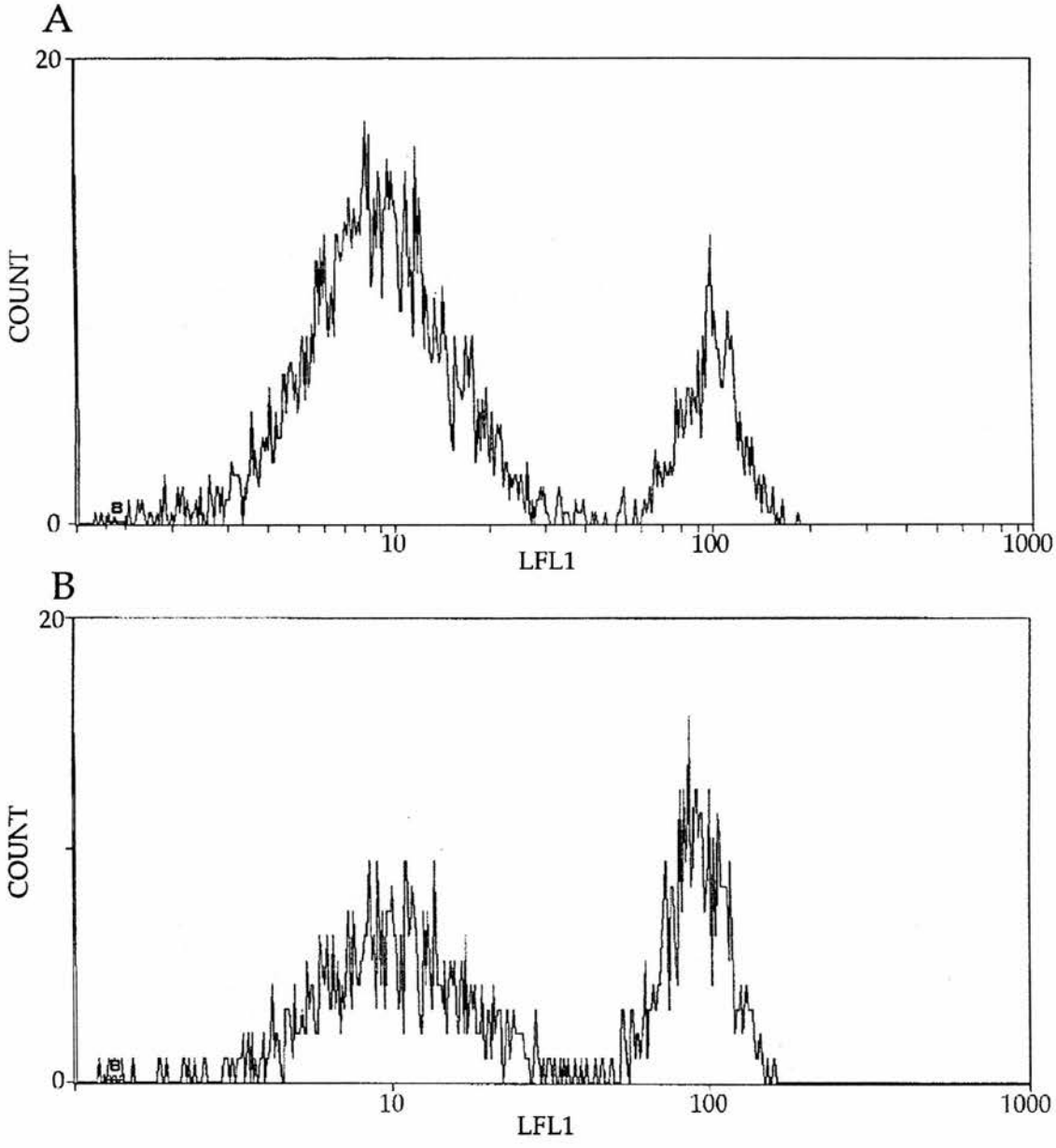

Figure 3.5 Effect of dexamethasone on neutrophil apoptosis as assessed by the levels of expression of surface CD16.
The results are representative flow cytometric traces of control cells *(A)* and cells treated with 1 μM dexamethasone *(B)*.

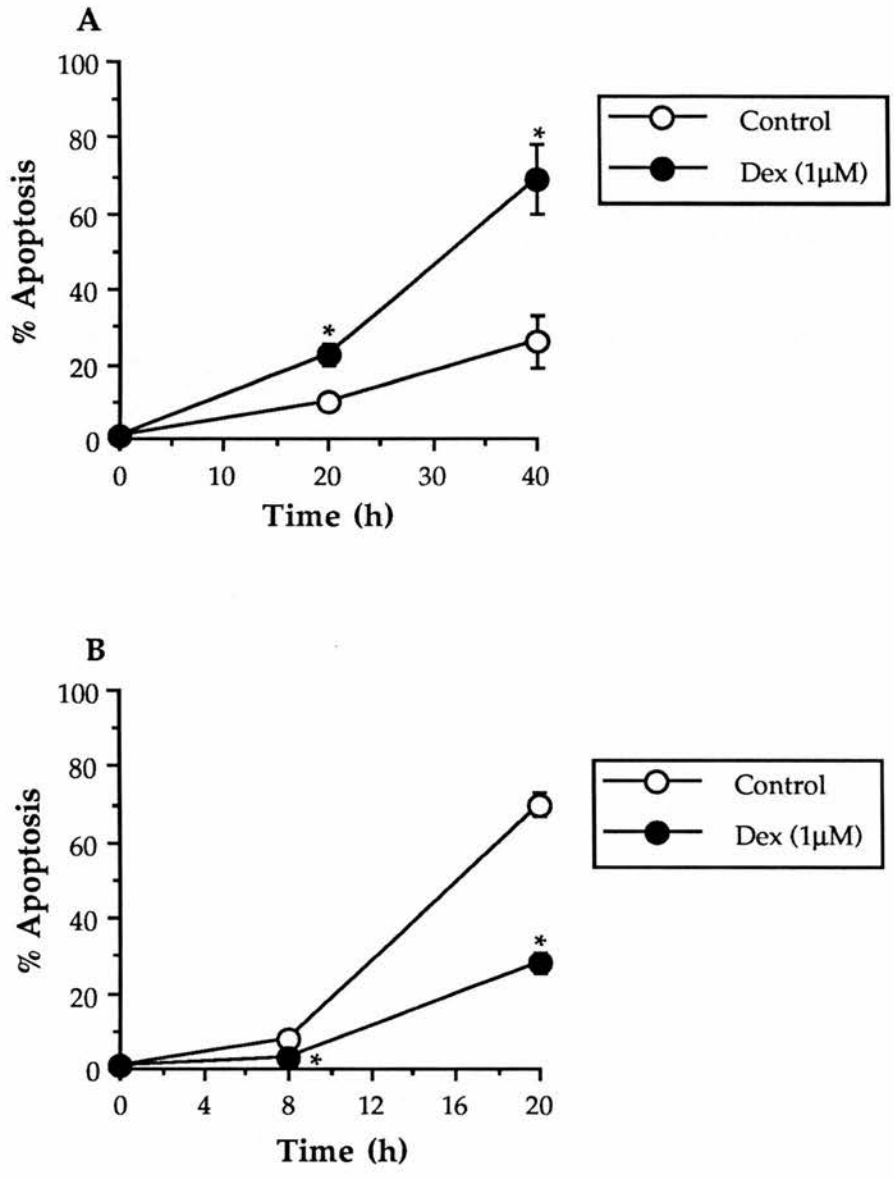

Figure 3.6 Time-course for the effect of dexamethasone on eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (1 μM). At the time periods indicated, the cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 10 independent experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (1 μM). At the time periods indicated, the cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 19 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

3.2.4 Concentration-dependency of the effect of dexamethasone on eosinophil and neutrophil apoptosis

The differential effect of dexamethasone on eosinophil and neutrophil apoptosis was concentration-dependent. Figure 3.7*A* illustrates an increase in the proportion of eosinophils exhibiting classical morphological features of apoptosis with increasing concentrations of dexamethasone at 0.01 to 1 μM. Figure 3.7*B* shows decreasing levels of neutrophil apoptosis in the presence of increasing concentrations of dexamethasone (0.01-1 μM).

3.2.5 Requirement for protein synthesis in glucocorticoid-mediated inhibition of neutrophil apoptosis

Treatment of neutrophils with cycloheximide, a protein synthesis inhibitor, rapidly induces apoptosis (Haslett et al., 1990), as shown in figure 3.8. To explore the possible involvement of protein synthesis in glucocorticoid-mediated inhibition of neutrophil apoptosis, cycloheximide was co-incubated with dexamethasone (figure 3.8*A*) and the alternative glucocorticoid, budesonide (figure 3.8*B*). These data show that cycloheximide (50 μM) abrogated glucocorticoid-mediated inhibition of neutrophil apoptosis. However, since the concentration of cycloheximide (50 μM) employed promoted the rate of neutrophil apoptosis when used alone, it is difficult to dissect the effect of protein synthesis inhibition from the glucocorticoid effect. To compare the efficacy of the two synthetic glucocorticoids used, the IC_{50} values of dexamethasone and budesonide were obtained from standard steroid binding assays and appropriate concentrations for use calculated based on the efficacy of dexamethasone (1 μM) as standard, since this was the principle glucocorticoid studied.

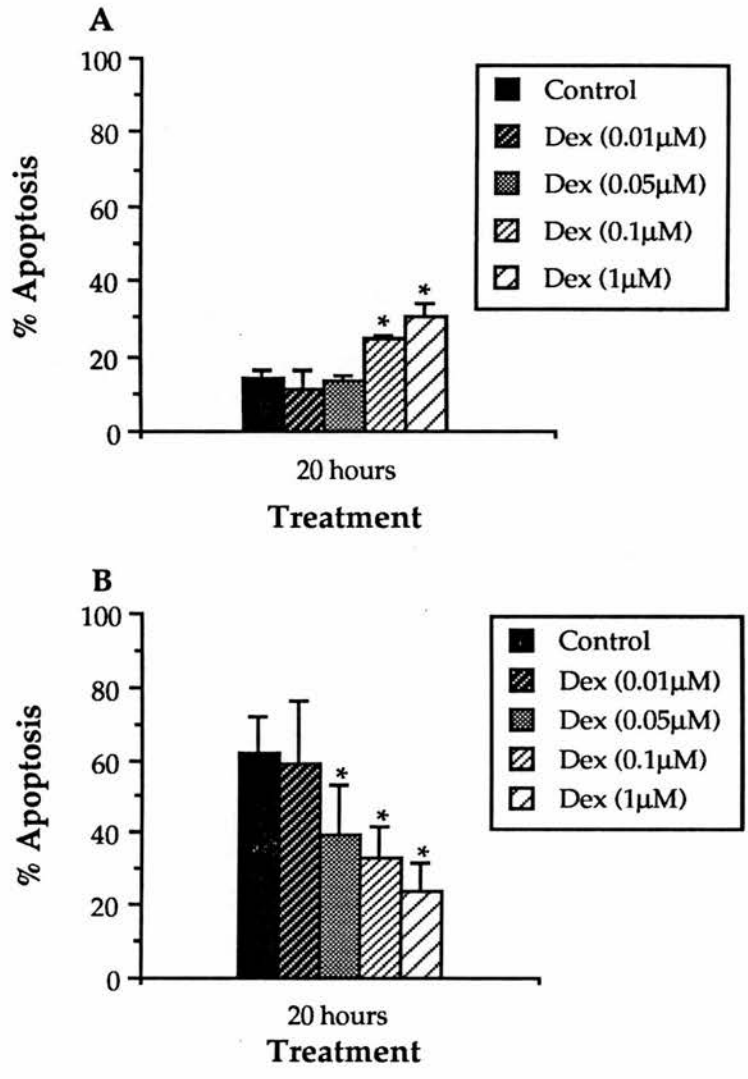

Figure 3.7 Concentration-response for the effect of dexamethasone on eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (0.01-1 μM). Eosinophils were harvested following 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dexamethasone (0.01-1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

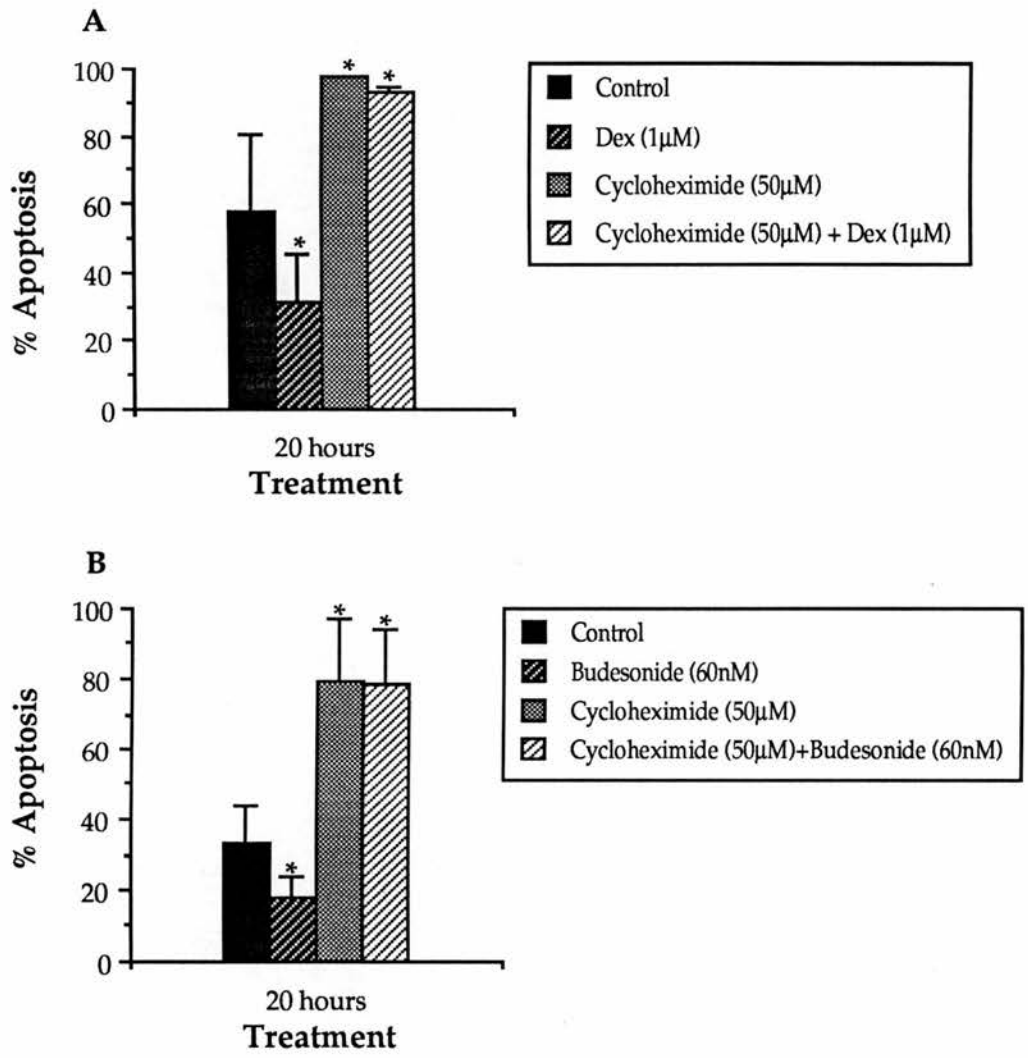

Figure 3.8 Effect of cycloheximide on basal, dexamethasone- and budesonide-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with cycloheximide (50 μM), in the presence and absence of either *(A)* dexamethasone (1 μM) or *(B)* budesonide (60 nM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

3.2.6 Effect of GM-CSF neutralising antibody on dexamethasone-mediated eosinophil and neutrophil apoptosis

Both eosinophils and neutrophils can produce GM-CSF (Kita et al., 1991a), a potent modulator of myeloid cell apoptosis. To investigate whether dexamethasone regulated apoptosis by modulating endogenous production of GM-CSF, a specific anti-GM-CSF mAb was used at a concentration sufficient to completely block the effect of 5 U/ml of GM-CSF. As illustrated in figure 3.9, GM-CSF neutralising Ab (25 μg/ml) abrogated GM-CSF (5 U/ml)-mediated inhibition of neutrophil apoptosis. This concentration of anti-GM-CSF mAb failed to block either the apoptosis-promoting effect of dexamethasone on eosinophils (figure 3.10*A*) or its apoptosis-inhibiting effect upon neutrophils (figure 3.10*B*). These data argue strongly against a role for GM-CSF as the mediator of the dexamethasone effect.

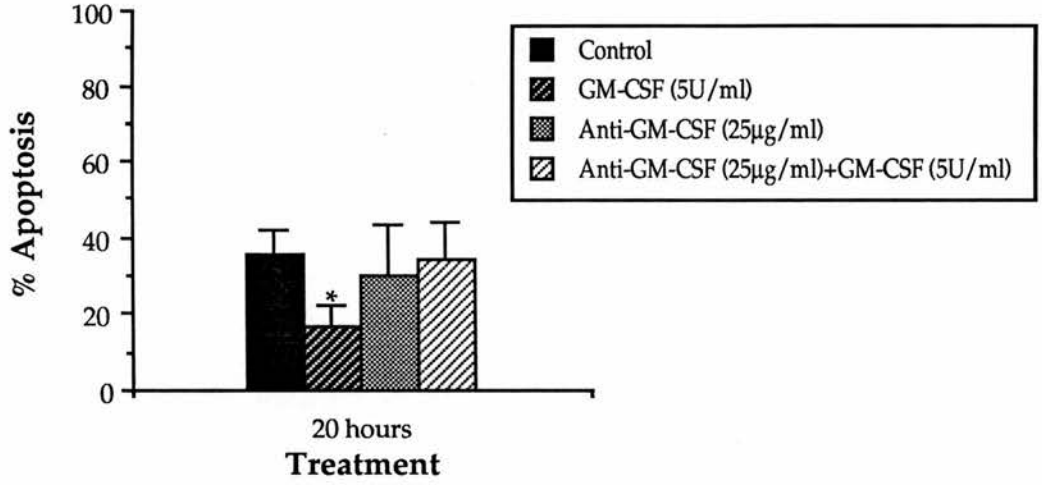

Figure 3.9 Effect of GM-CSF neutralising antibody on basal and GM-CSF-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured for 20 h either in serum-supplemented Iscove's DMEM alone (control), GM-CSF (5 U/ml), mouse anti-human GM-CSF neutralising mAb (25 µg/ml) or an identical concentration of GM-CSF pre-incubated for 30 min at 37°C with the neutralising mAb (25 µg/ml). Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

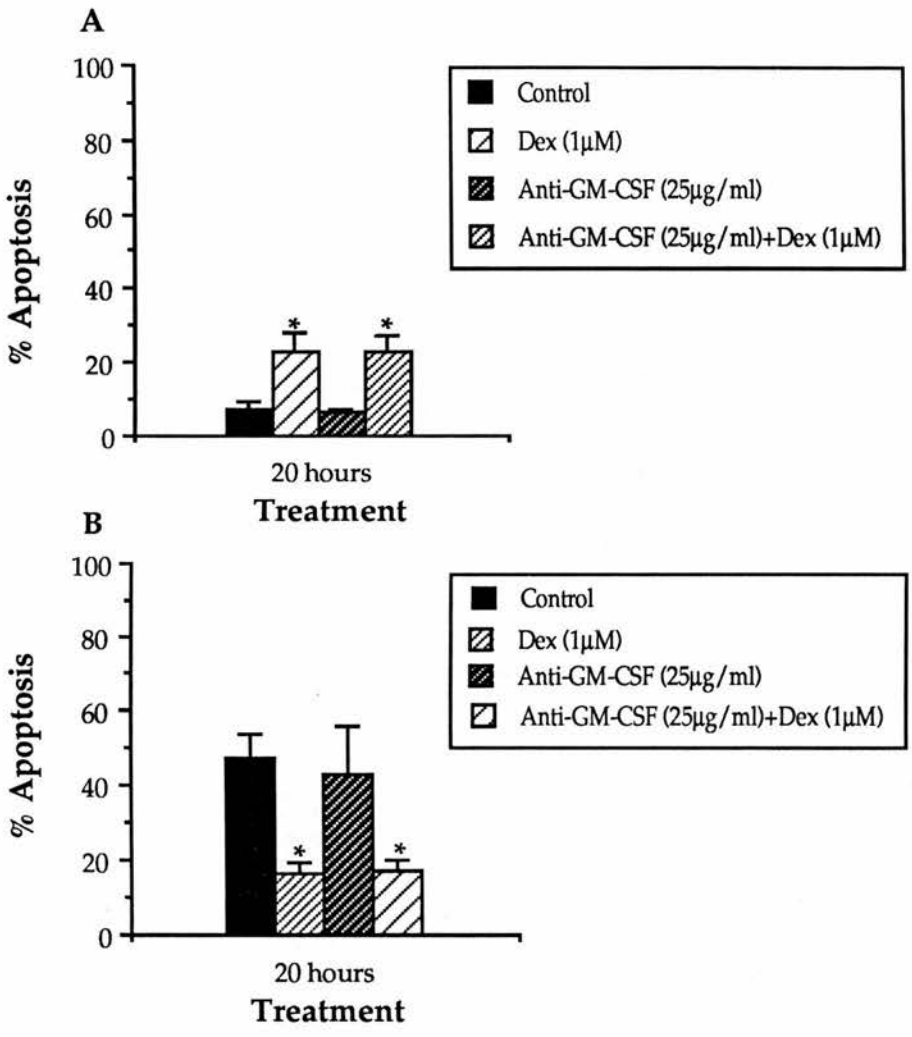

Figure 3.10 Effect of GM-CSF neutralising antibody on basal and dexamethasone-mediated eosinophil (A) and neutrophil (B) apoptosis.
A, Eosinophils ($2x10^6$/ml) were incubated either in serum-supplemented Iscove's DMEM alone (control), dexamethasone (1 μM), mouse anti-human GM-CSF neutralising mAb (25 μg/ml) or an identical concentration of dexamethasone pre-incubated for 30 min at 37°C with the neutralising mAb (25 μg/ml). Eosinophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-6 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were incubated either in serum-supplemented Iscove's DMEM alone (control), dexamethasone (1 μM), mouse anti-human GM-CSF neutralising mAb (25 μg/ml) or an identical concentration of dexamethasone pre-incubated for 30 min at 37°C with the neutralising mAb (25 μg/ml). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-6 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

3.3 Discussion

We have demonstrated that [illegible] ametrically opposed effects upon the rate of [illegible] *in vitro*; promoting eosinophil apoptosis and inhibiting neutrophil apoptosis. Administration of glucocorticoids has been reported to induce fluctuations in the numbers of peripheral leukocytes *in vivo*, observations which have long indicated divergent responses among granulocyte types (Schleimer, 1990). The rapid and dramatic peripheral blood eosinopenia following glucocorticoid treatment (Saunders & Adams, 1950) is not mirrored in neutrophil counts, which, in fact, show an increase (Athens et al., 1961, Dale et al., 1975). However, the mechanisms underlying these phenomena have not been fully elucidated. We propose that the direct influence of glucocorticoids on apoptosis that we have described may be a major factor in the production of this effect.

Dexamethasone-induced alterations in the rate of granulocyte apoptosis have been shown to be mediated by the glucocorticoid receptor (GR) (Meagher et al., 1996, Cox, 1995). These experiments involved the GR antagonist, RU38486, which is a synthetic glucocorticoid that binds to the GR without producing agonist activity (Bourgeois et al., 1984). The presence of RU38486 alone was shown to have no effect on the rate of apoptosis in either eosinophils (Meagher et al., 1996) or neutrophils (Meagher et al., 1996, Cox, 1995), arguing against the possibility of toxicity by RU38486 and suggesting that endogenous glucocorticoids (present in serum) are not involved in determining the constitutive rate of apoptosis of these cells in culture. Co-culture of eosinophils or neutrophils with 1 μM dexamethasone and 10 μM RU38486 restored the rate of apoptosis in

each culture to control levels (Meagher et al., 1996). We have also shown that other glucocorticoid effects are also mediated by the GR, and these data are discussed in Chapters 6 and 7. Similarly, Cox and colleagues have demonstrated a concentration-dependent attenuation of dexamethasone-mediated inhibition of neutrophil apoptosis in the presence of RU38486, with concentrations of RU38486 as low as 0.1 μM being shown to significantly abrogate the dexamethasone effect (Cox, 1995). Moreover, the inhibitory effect of RU38486 was shown to be specific and did not alter either GM-CSF-induced inhibition of apoptosis or cycloheximide-induced promotion of apoptosis (Meagher et al., 1996). Furthermore, in agreement, Cox and colleagues have also demonstrated that RU38486 at a concentration (1 μM) sufficient to attenuate dexamethasone-mediated inhibition of neutrophil apoptosis, did not alter LPS-stimulated survival (Cox, 1995). Collectively, these data illustrate that RU38486 effectively and specifically inhibits dexamethasone-induced alterations in the rate of granulocyte apoptosis, which are directly mediated by the GR.

We have also shown that the alternative synthetic glucocorticoid budesonide, which is available for clinical use in an inhaled form in Canada and Europe, mirrors the effect of dexamethasone on neutrophil apoptosis (figure 3.8*B*). Furthermore, endogenous glucocorticoids, such as cortisol, have been shown to promote eosinophil apoptosis and inhibit neutrophil apoptosis, in contrast to progesterone, which has little glucocorticoid activity and does not modulate granulocyte apoptosis (Meagher et al., 1996). Similarly, several other laboratories have also demonstrated that the ability of dexamethasone to inhibit neutrophil apoptosis is confined to glucocorticoids, with the relative survival-promoting effects been shown to correlate with their relative clinical

potencies (Cox, 1995, Liles et al., 1995). Collectively, these observations illustrate that dexamethasone-mediated up-regulation of apoptosis in eosinophils and down-regulation of apoptosis in neutrophils is mirrored by the physiologic glucocorticoid, cortisol, and indicate that this modulation of granulocyte apoptosis is limited to glucocorticoids and is not simply a nonspecific response to high concentrations of any steroid.

Pharmacological inhibitors of protein synthesis have been widely used to examine how apoptosis is regulated. It has been reported that many glucocorticoid effects are protein synthesis dependent. For example, apoptosis that is induced in thymocytes by dexamethasone is attenuated by co-treatment with cycloheximide, a translation inhibitor, indicating that this dexamethasone-mediated promotion of apoptosis is dependent on the synthesis of new proteins (Wyllie et al., 1984). In contrast, treatment of both neutrophils (figure 3.8, Haslett et al., 1990) and eosinophils (Meagher and Haslett, unpublished observations) with cycloheximide rapidly induces apoptosis, indicating that granulocyte apoptosis does not depend on new protein synthesis. These observations suggest that eosinophil and neutrophil apoptosis may be normally held in check by the constant production of an inhibitor protein, the production of which may be differentially affected by treatment with dexamethasone in the two cell types. Studies investigating the dependence on protein synthesis of dexamethasone in differentially regulating granulocyte apoptosis have been hampered by the fact that the effects of glucocorticoids on eosinophil and neutrophil apoptosis are not readily apparent within 8 h of treatment, by which time, 50 μM cycloheximide has induced apoptosis in typically 80% of both eosinophils and neutrophils. It has therefore been difficult to dissect the effect of

protein synthesis inhibition from the glucocorticoid effect at this time. However, Cox and colleagues have now reported that much lower concentrations of cycloheximide (1 μg/ml ≡ 28 nM) are sufficient to interfere with, but not abolish, protein synthesis in neutrophils (Cox et al., 1994). Thus, several more recent reports have now investigated whether dexamethasone-mediated inhibition of neutrophil apoptosis is protein synthesis dependent. Kato and colleagues have demonstrated that cycloheximide, at a concentration that did not affect neutrophil apoptosis when used alone (0.1 μg/ml ≡ 2.8 nM), completely abrogated dexamethasone-mediated inhibition of neutrophil apoptosis (Kato et al., 1995). Similarly, identical data has also been presented using a 1 μg/ml concentration of cycloheximide (Cox & Austin, 1997). These findings lend further support to the hypothesis that glucocorticoid hormones enhance the production of apoptosis inhibitory proteins in neutrophils.

Glucocorticoids have been reported to modulate cytokine production from various cell types, including monocytes (Lew et al., 1988, Waage & Bakke, 1988) and murine T lymphocytes (Culpepper & Lee, 1985; 1987). There have been a number of examples of a stimulation of inflammatory cell survival inducing production of a survival factor that is released into the supernatant, where it acts on adjacent cells in an autocrine or paracrine fashion. For example, Lamas et al., (1989) have demonstrated that dexamethasone treatment of endothelial cell cultures markedly inhibited eosinophil survival induced by supernatant generated by those cells. Moreover, Cox and colleagues have illustrated that conditioned medium generated from human bronchial epithelial cells (HBEC) can support eosinophil survival via the elaboration of GM-CSF. In addition, it was shown that the glucocorticoid, budesonide, inhibited both

unstimulated and IL-1-stimulated GM-CSF production by HBEC and had a direct effect on eosinophils, since it prevented the ability of these cells to respond to GM-CSF (Cox et al., 1991). Thus it has been suggested that steroids may modulate this process by inhibiting cytokine production from HBEC and by a direct effect on eosinophils preventing their response to cytokines. Furthermore, several groups have reported that glucocorticoids inhibit the effects of IL-5 and GM-CSF in prolonging eosinophil survival *in vitro* (Her et al., 1991, Lamas et al., 1991, Wallen et al., 1991, Hallsworth et al., 1992). Interestingly, glucocorticoids potentiate GM-CSF-mediated inhibition of neutrophil apoptosis *in vitro* (Meagher et al., 1996, Cox, 1995). However, although these findings are of interest since glucocorticoids are used therapeutically at times when stimuli such as GM-CSF might also be present, it is difficult to draw any mechanistic conclusions from such experiments.

Upon appropriate activation, both eosinophils and neutrophils are capable of producing and releasing GM-CSF (Kita et al., 1991a) which is a potent inhibitor of apoptosis in both cell types (Stern et al., 1992, Lee et al., 1993). We were interested to investigate whether glucocorticoids exerted diametrically opposed effects upon myeloid cell apoptosis through differential regulation of the elaboration of GM-CSF. Eosinophils and neutrophils were routinely isolated according to methods designed to minimise cell activation (Stern et al., 1992, Savill et al., 1989a). We have reported that GM-CSF was undetectable in aging cultures and remained undetectable following glucocorticoid treatment (Meagher et al., 1996). Further studies demonstrated that an excess of blocking anti-GM-CSF Ab failed to affect the modulation of apoptosis caused by glucocorticoids (figure 3.10). In agreement with our findings, Kato and colleagues have

reported that addition of supernatants obtained from neutrophils pretreated with dexamethasone did not inhibit neutrophil apoptosis (Kato et al., 1995). Contradicting these findings, Cox & Austin (1997) have recently reported that dexamethasone-conditioned medium induced neutrophil survival comparable to that found with dexamethasone. However, this study also demonstrated that addition of RU38486 completely inhibited the effects of dexamethasone-conditioned medium so that survival was reduced to levels similar to those found in control conditions, suggesting that dexamethasone does not induce the release of stable factors that modulate neutrophil apoptosis in an autocrine or paracrine fashion. We, therefore, propose that the differential regulation of granulocyte apoptosis by glucocorticoids is not mediated by GM-CSF, and the exact mechanism of action remains to be elucidated.

The unique nature appertaining to the effect of glucocorticoids on neutrophil apoptosis has been illustrated by Cox (1995), who has demonstrated that the effect on cell survival is distinct from priming or activation of the cell. This report shows that cells induced to survive by dexamethasone retain the capacity to generate superoxide without significant enhancement of this response either in the basal state or following FMLP stimulation. Moreover, untreated cells produced detectable levels of IL-8, which is an index of new protein synthesis by activated cells, whereas dexamethasone treatment significantly inhibited this cytokine production. These results are particularly intriguing since all other stimuli including GM-CSF, LPS and C5a, known to inhibit neutrophil apoptosis also cause cell activation to some degree (Brach et al., 1992, Colotta et al., 1992, Balazovich et al., 1991). The ability of dexamethasone to prolong neutrophil survival without induction of cell

activation would enhance the neutrophils host defence capabilities but limit their potential to exacerbate the inflammatory response through production of chemotactic and activating signals (Lloyd & Oppenheim, 1992).

Demonstration of the marked apoptosis-promoting effects of dexamethasone on eosinophil apoptosis *in vitro* may underlie the known beneficial effects of glucocorticoids on established 'eosinophilic' inflammatory diseases such as allergy and asthma. In agreement with our findings, Woolley and colleagues have recently provided the first report of eosinophil apoptosis *in vivo,* in human asthma and have highlighted the promotion of apoptosis after corticosteroid treatment, concurrent with improved lung function (Woolley et al., 1996). These findings provide direct human *in vivo* support for the dexamethasone-mediated up-regulation of eosinophil apoptosis observed *in vitro* and indicate that apoptosis-induction is clinically relevant to asthma. However, experience in the clinical situation has not demonstrated the same beneficial effects of glucocorticoids in neutrophil-mediated inflammatory diseases, such as chronic obstructive airways disease (Stoller et al., 1987). The opposite effect of glucocorticoids on neutrophil apoptosis *in vitro* may therefore explain the lower efficacy of these drugs in 'neutrophilic' inflammatory diseases. The differential effect of glucocorticoids on granulocyte apoptosis provides the ability to selectively induce apoptosis in discrete populations of inflammatory cells and may serve as a powerful tool improving the therapeutic approach to allergic inflammation.

Chapter 4

The role of Ca^{2+} and PKC in basal and glucocorticoid-mediated granulocyte apoptosis

4.1 Introduction

There has been intense interest in the underlying intracellular regulatory signalling mechanisms mediating apoptosis. Several familiar signal transduction pathways have now been implicated in the positive and negative control of apoptosis in cells of diverse tissue origin. In particular, considerable attention has focussed on the role of Ca^{2+} and PKC since it is well known that various physiological responses can result from synergistic interactions between this network of intracellular signalling systems.

Several lines of evidence indicate that alterations in the cytosolic Ca^{2+} concentration are involved in the regulation of apoptosis. Early work by Kaiser & Edelman (1977; 1978) demonstrated that glucocorticoid-stimulated apoptosis is associated with Ca^{2+} influx and that the cytolytic effects of glucocorticoids on lymphoid cells can be mimicked by treating the cells with Ca^{2+} ionophores. It has since been well documented that glucocorticoids induce cytosolic Ca^{2+} elevation in thymocytes and that the Ca^{2+} increase is responsible for the initiation of apoptosis (Cohen & Duke, 1984, McConkey et al., 1989a). Treatment with thapsigargin and Ca^{2+} ionophores has been shown to induce apoptosis in thymocytes (Cohen & Duke, 1984, McConkey et al., 1989b, Jiang et al., 1994) and in other cell types including human hepatoma cells (Kaneko & Tsukamoto, 1994), cerebellar granule neurones (Levick et al., 1995), rat prostatic glandular

cells (Martikainen & Isaacs, 1990) and androgen-independent prostatic cancer cells (Martikainen et al., 1991, Furuya et al., 1994). Moreover, certain chemical toxins may also promote apoptosis by disrupting intracellular Ca^{2+} homeostasis, leading to nonphysiological Ca^{2+} increases that promote endonuclease activation and apoptotic cell death (McConkey et al., 1988, Aw et al., 1990). Furthermore, Ca^{2+} increases have been observed in many other examples of apoptosis (Perotti et al., 1990, McConkey et al., 1991, Zheng et al., 1991, Bellomo et al., 1992). Direct evidence that Ca^{2+} increases can mediate apoptosis has been obtained from experiments with intracellular Ca^{2+} buffering agents and extracellular Ca^{2+} chelators, which have been shown to inhibit both DNA fragmentation and death in apoptotic cells (McConkey et al., 1989a, McConkey et al., 1989c, Aw et al., 1990, McConkey et al., 1990a, Perotti et al., 1990, McConkey et al., 1991, Bellomo et al., 1992, Story et al., 1992, Robertson et al., 1993). Furthermore, calmodulin antagonists have been shown to interfere with apoptosis in some of these systems (McConkey et al., 1989a, Dowd et al., 1991) and increases in calmodulin expression are linked to apoptosis in glucocorticoid-treated WEH17.2 lymphocytes (Dowd et al., 1991) and in the prostate following withdrawal of androgen (Furuya & Isaacs, 1993). Independent evidence for the involvement of Ca^{2+} influx in the triggering of apoptosis has come from studies with specific Ca^{2+} channel blockers, which abrogate apoptosis in the regressing prostate following testosterone withdrawal (Martikainen & Isaacs, 1990) and in pancreatic β cells treated with serum from patients with type I diabetes (Juntti-Berrgren et al., 1993). Finally, recent work on the biochemical mechanisms of apoptosis suppression by the bcl-2 oncoprotein have pointed to a central role for Ca^{2+} in regulating apoptosis, in particular in the regulation of intracellular Ca^{2+}

compartmentalisation (Baffy et al., 1993, Lam et al., 1994, Marin et al., 1996). Thus, elevation of $[Ca^{2+}]_i$ appears to represent a relatively common trigger for apoptosis in cells of diverse tissue origins.

In other cellular systems, however, increases in cytosolic Ca^{2+} inhibit apoptotic cell death. For example, Ca^{2+} ionophores block apoptosis in aged neutrophils (Whyte et al., 1993b). In addition, treatment of IL-3-dependent BAF-3 cells (a murine bone marrow progenitor cell line) with Ca^{2+} ionophores prevents endogenous endonuclease activation and cell death following withdrawal of IL-3 (Rodriguez-Tarduchy et al., 1990).

Similarly, PKC has also been implicated in both the positive and negative regulation of apoptosis. PKC activation has been shown to stimulate apoptosis in thymocytes (Kizaki et al., 1989a), U937 cells (De Vente et al., 1995a) and in MCF-7 breast cancer cells (De Vente et al., 1995b). Furthermore, it has been reported that glucocorticoid-induced apoptosis in thymocytes appears to be dependent on PKC, since it is inhibited by PKC inhibitors (Ojeda et al., 1990).

Other work suggests that PKC activation can also inhibit apoptosis. Phorbol esters and other activators of PKC have been shown to inhibit apoptosis in a variety of cell types including thymocytes (McConkey et al., 1989d, Tomei et al., 1988, Kizaki et al., 1989b), a human mammary adenocarcinoma cell line (BT-20) (Bellomo et al., 1992), normal (Illera et al., 1993) and leukemic (McConkey et al., 1991, Forbes et al., 1992) B cells, human synovial cells (Perotti et al., 1990), IL-3 dependent haematopoietic cells (Rajotte et al., 1992) and kidney epithelial cells (Koseki et al., 1992).

In light of the current literature, we investigated the direct effects of agents that increase intracellular Ca^{2+} levels and inhibit PKC on the constitutive and glucocorticoid-mediated rate of eosinophil and neutrophil apoptosis.

4.2 Results

4.2.1 Concentration-dependency of the effect of A23187 on neutrophil apoptosis

Exposure of neutrophils to the calcium ionophore, A23187 (0.01-0.1 μM), produced a concentration-dependent inhibition in the rate of apoptosis (Figure 4.1). Assessment of cell viability and recovery demonstrated that A23187 did not significantly alter either of these parameters, at the concentrations shown in figure 4.1. However, higher concentrations of A23187 (1-10 μM) rendered the cells necrotic, as assessed by trypan blue exclusion. From these data, a maximal concentration of A23187 (0.1 μM) was selected for use in further studies.

4.2.2 Divergent effects of agents that increase intracellular Ca^{2+} on eosinophil and neutrophil apoptosis

Eosinophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM, in the presence of A23187 (0.1 μM) and thapsigargin (2 μM), showed an increase in the light microscopic features of apoptosis at 20 h, compared with the control population (figure 4.2*A*). In contrast to the effects observed on eosinophils, neutrophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM showed decreased apoptosis in the presence of both A23187 (0.1 μM) and thapsigargin (2 μM), as illustrated in figure 4.2*B*. At the concentrations used these intracellular Ca^{2+} elevating agents did not affect cell viability or recovery in either granulocyte type. Interestingly, the pro-apoptotic effect seen in eosinophils and the anti-apoptotic effect seen in neutrophils was more pronounced upon treatment with thapsigargin compared with the effects seen in the presence of A23187.

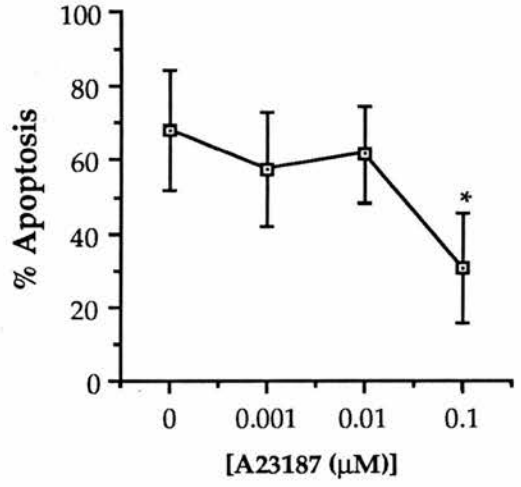

Figure 4.1 Concentration response for the effect of A23187 on neutrophil apoptosis.

Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or in the presence of A23187 (0.01-0.1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

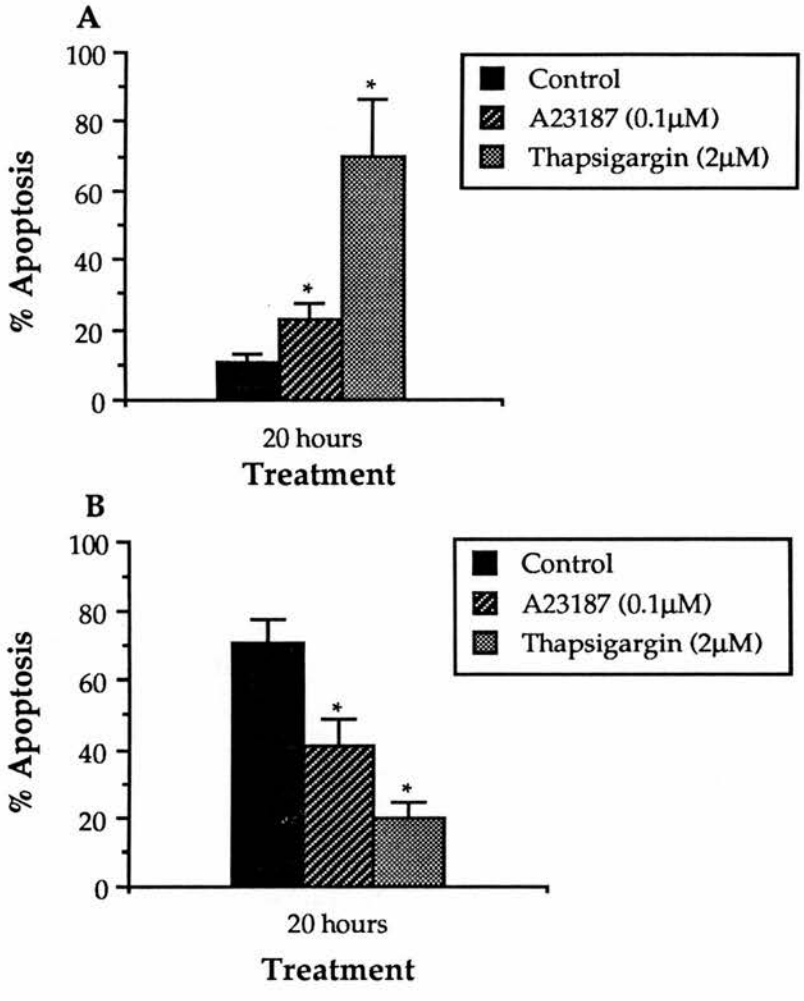

Figure 4.2 Effect of A23187 and thapsigargin on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured for 20 h in serum-supplemented Iscove's DMEM either alone (control) or in the presence of A23187 (0.1 μM) and thapsigargin (2 μM). Eosinophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4-7 separate experiments, each performed in duplicate (*$p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured for 20 h in serum-supplemented Iscove's DMEM either alone (control) or in the presence of A23187 (0.1 μM) and thapsigargin (2 μM). Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-7 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

4.2.3 Role of Ca^{2+} in dexamethasone-mediated eosinophil and neutrophil apoptosis

The differing effects of A23187 and thapsigargin on granulocyte apoptosis mirror the effect of dexamethasone on these cell types (Chapter 3); promoting eosinophil, but inhibiting neutrophil apoptosis. To investigate whether dexamethasone modulated apoptosis by inducing an elevation in intracellular Ca^{2+}, A23187 (0.1 μM) and thapsigargin (2 μM) were co-cultured in the presence of dexamethasone (1 μM). Figure 4.3 illustrates that A23187 and thapsigargin further enhance the eosinophil apoptosis-promoting and neutrophil apoptosis-inhibiting ability of dexamethasone. However, it is difficult to draw precise mechanistic conclusions from such experiments, given that all of these agents individually modulate the rate of basal granulocyte apoptosis in culture. An alternative strategy was therefore adopted involving BAPTA/AM, an intracellular Ca^{2+} chelator. Figure 4.4 illustrates that BAPTA/AM (2.5 μM) promotes neutrophil apoptosis after 8 h in culture. The assessment of apoptosis was carried out at an earlier time period (8 h) to enable easy detection, in comparison with the usual later time of 20 h, where a greater proportion of cells would have undergone the apoptotic process. BAPTA/AM (2.5 μM) and dexamethasone (1 μM) were co-cultured, to determine whether chelation of intracellular Ca^{2+} abrogated the anti-apoptotic effect of dexamethasone, as observed after co-incubation of BAPTA/AM (2.5 μM) and A23187 (0.1 μM). Figure 4.4 illustrates that chelation of intracellular Ca^{2+} does not affect dexamethasone-mediated inhibition of neutrophil apoptosis, after 8 h in culture.

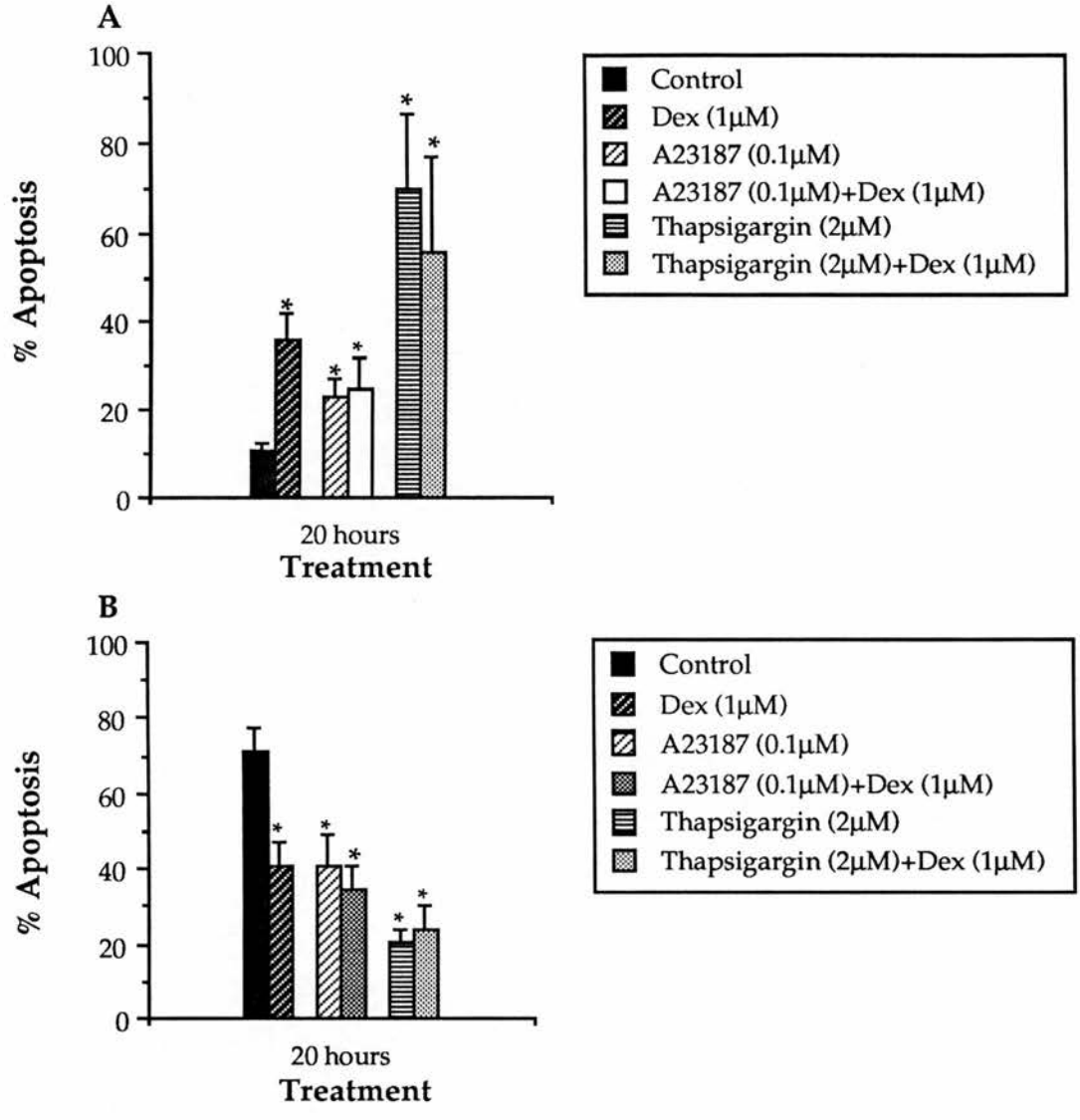

Figure 4.3 Effect of A23187 and thapsigargin on dexamethasone-mediated eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with A23187 (0.1 μM) and thapsigargin (2 μM), in the presence and absence of dexamethasone (1 uM). Eosinophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 2-7 separate experiments, each performed in duplicate (*$p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with A23187 (0.1 μM) and thapsigargin (2 μM), in the presence and absence of dexamethasone (1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-7 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

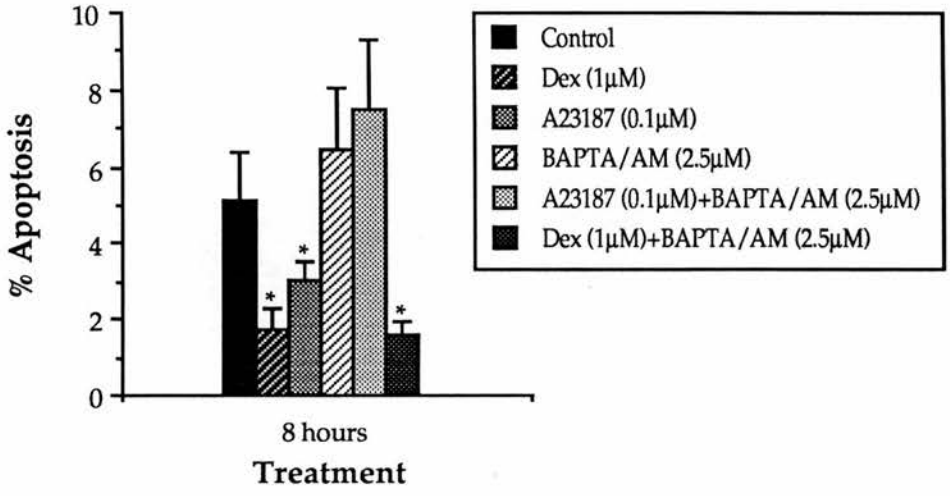

Figure 4.4 Effect of BAPTA/AM on basal, A23187- and dexamethasone-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with BAPTA/AM (2.5 μM), in the presence and absence of A23187 (0.1 μM) or dexamethasone (1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

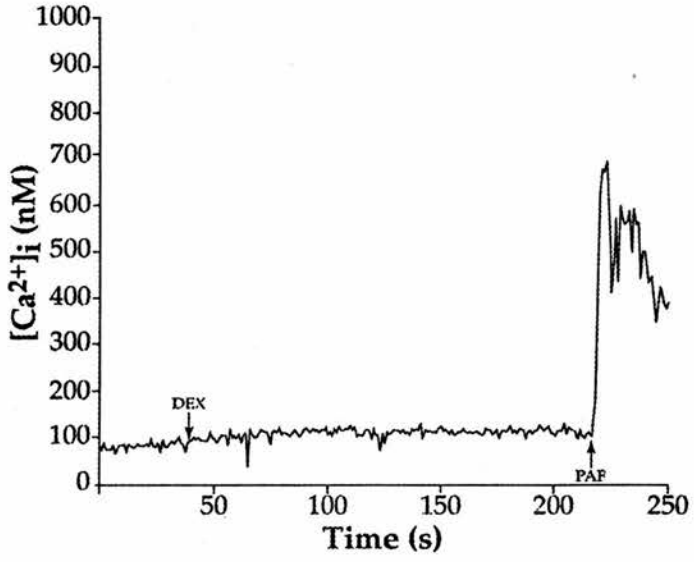

Figure 4.5 Measurement of cytosolic calcium levels in freshly isolated neutrophils in response to different stimuli.
Cytosolic calcium measurements (nM) in fura-2-loaded, freshly isolated neutrophils ($2x10^6$/ml) over 250 s. Dexamethasone (1 μM) was added to the cell suspension after 40 s, followed by the sequential addition of PAF (100 nM), after 225 s. The results presented are from a representative experiment.

Cytosolic calcium levels, $[Ca^{2+}]_i$, were measured using the Ca^{2+}-sensitive fluorescent dye fura-2/AM in freshly isolated neutrophils, with the subsequent addition of dexamethasone (1 μM) and PAF (100 nM), as indicated in figure 4.5. Dexamethasone (1 μM) did not induce an elevation in $[Ca^{2+}]_i$ above resting $[Ca^{2+}]_i$ values of 100 nM or below, which are consistent with basal $[Ca^{2+}]_i$ levels found in other studies (Whyte et al., 1993b). In contrast, addition of PAF (100 nM) to the cuvette, induced a rapid and dramatic rise in $[Ca^{2+}]_i$ to a peak of 700 nM, before returning to control levels. Thus, figure 4.5 illustrates that dexamethasone does not induce a rapid transient rise in $[Ca^{2+}]_i$ in freshly isolated neutrophils.

4.2.4 Effect of PKC inhibitors on eosinophil and neutrophil apoptosis

Eosinophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM, in the presence of the PKC inhibitor staurosporine (1 μM), showed an increase in the light microscopic features of apoptosis at 20 h, compared with the control population (figure 4.6), without significantly affecting either cell viability or recovery. Figure 4.7 illustrates the effect of staurosporine *(A)* and a more specific PKC inhibitor, Ro-31-8220 *(B)* on the rate of neutrophil apoptosis, after 4, 8 and 20 h in culture. The highest concentration of staurosporine used (10 μM), promoted neutrophil apoptosis to a value comparable with that seen in the presence of Ro-31-8220 (10 μM). The results observed in the presence of lower concentrations of staurosporine (0.1-1 μM), were inconsistent between the various time periods of apoptosis assessment and did not show any consistent trend (figure 4.7*A*). However, in the presence of Ro-31-8220 (0.1-10 μM), the proportion of neutrophils exhibiting classical morphological features of apoptosis increased in a concentration-dependent manner, with a consistent increasing trend observed at all time periods investigated (figure 4.7*B*). These results indicate that inhibition of PKC, promotes the rate of both eosinophil and neutrophil apoptosis.

4.2.5 Involvement of PKC in modulating dexamethasone-mediated inhibition of neutrophil apoptosis

At a concentration of 0.1 μM staurosporine and Ro-31-8220 were shown to have no significant effect upon the control rate of neutrophil apoptosis (figure 4.7). To investigate whether dexamethasone-mediated inhibition of neutrophil apoptosis involved PKC activation, neutrophils were co-cultured with dexamethasone in the presence of staurosporine (figure

4.8*A*) and Ro-31-8220 (figure 4.8*B*) at a concentration of 0.1 μM for 20 h. Interestingly, we observed a partial abrogation of dexamethasone-mediated inhibition of neutrophil apoptosis, indicating that activation of PKC may be involved in the mechanism of this dexamethasone-induced effect.

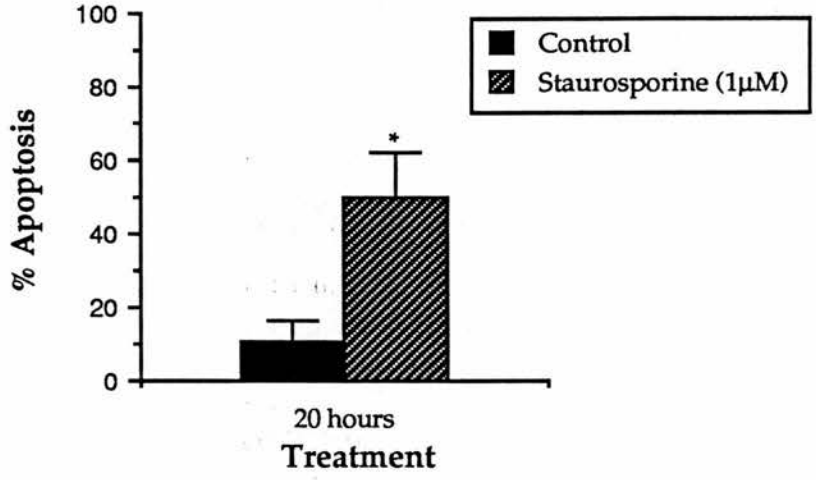

Figure 4.6 Effect of staurosporine on eosinophil apoptosis.
Eosinophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or in the presence of staurosporine (1 μM). Eosinophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (*$p<0.05$ compared with control values).

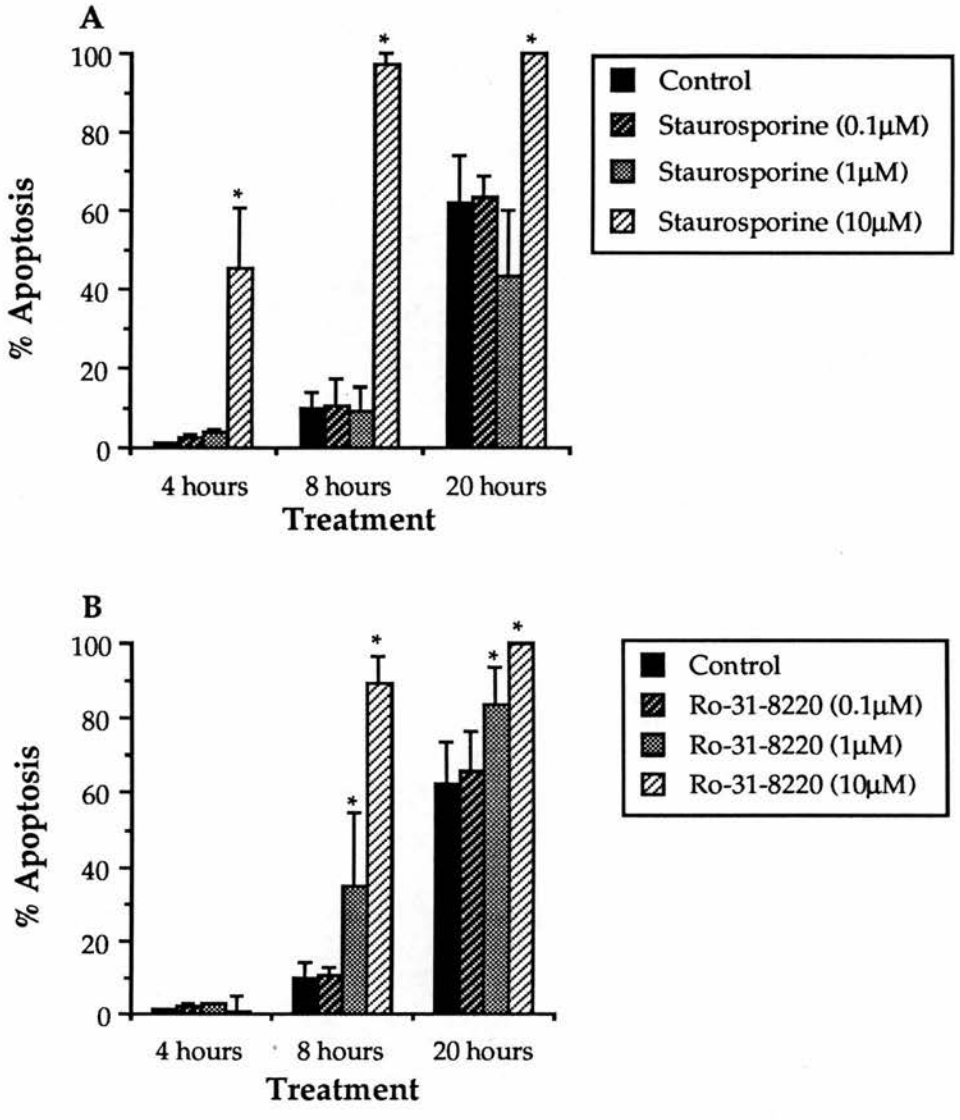

Figure 4.7 Effect of staurosporine and Ro-31-8220 on neutrophil apoptosis.

Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or in the presence of *(A)* staurosporine (0.1-10 μM) or *(B)* Ro-31-8220 (0.1-10 μM). Neutrophils were harvested following 4, 8 and 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (*$p<0.05$ compared with control values).

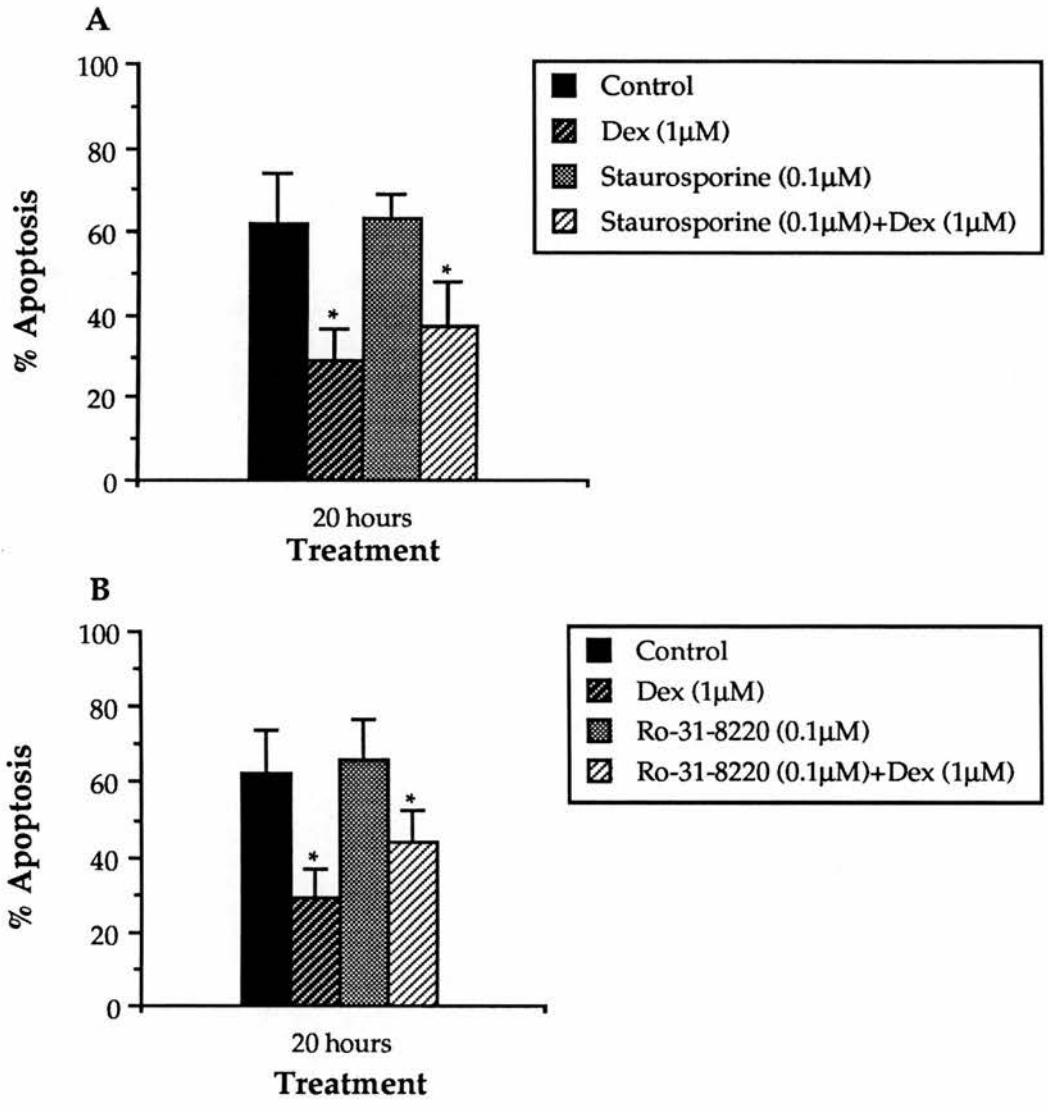

Figure 4.8 Effect of staurosporine and Ro-31-8220 on dexamethasone-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence or absence of *(A)* staurosporine (0.1 μM) or *(B)* Ro-31-8220 (0.1 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (*$p<0.05$ compared with control values).

4.3 Discussion

We have demonstrated that elevation of $[Ca^{2+}]_i$, by either the calcium ionophore A23187 or thapsigargin, differentially affects that rate of granulocyte apoptosis; promoting eosinophil and inhibiting neutrophil apoptosis (figure 4.2). Whyte and colleagues have previously reported that elevation of $[Ca^{2+}]_i$ exerts an inhibitory effect upon the rate of neutrophil apoptosis (Whyte et al., 1993b), however the promotion of eosinophil apoptosis observed after culture of eosinophils in the presence of either Ca^{2+} elevating agent, has, to our knowledge, not been previously reported. Moreover, we have illustrated that elevation of $[Ca^{2+}]_i$, through independent mechanisms, elicits consistent trends in the modulation of granulocyte apoptosis. However, the pro-apoptotic effect observed in eosinophils and the anti-apoptotic effect observed in neutrophils was more pronounced in the presence of thapsigargin as compared to A23187. These results may suggest that the mechanism of calcium elevation is an important factor in the regulation of granulocyte apoptosis. A23187 is a compound which has been shown to elevate $[Ca^{2+}]_i$ due to its Ca^{2+} ionophore ability (Reed & Lardy, 1972). However, the mechanism of thapsigargin-mediated $[Ca^{2+}]_i$ elevation involves the initial release of Ca^{2+} from intracellular stores, by a selective inhibition of the endoplasmic reticulum Ca^{2+}-ATPase and entry of Ca^{2+} from the extracellular space (Takemura et al., 1989, Thastrup et al., 1989). This capacitative Ca^{2+} entry pathway is now known to exist in most cell types (Putney, 1990). Thapsigargin-induced $[Ca^{2+}]_i$ elevation in cells is pharmacologically indistinguishable from that observed during agonist stimulation and mimics physiological $[Ca^{2+}]_i$ increases. This avoids the possible nonspecific effects associated with Ca^{2+} ionophores used in previous studies (Wyllie et al., 1984) and provides a specific approach to examine

the role of Ca^{2+} in the regulation of apoptosis. The more pronounced effects of $[Ca^{2+}]_i$ elevation upon granulocyte apoptosis in the presence of thapsigargin, as compared to A23187, may therefore indicate that the depletion of thapsigargin-sensitive endoplasmic reticulum Ca^{2+} pools is important in the mechanism of $[Ca^{2+}]_i$ elevation regulating granulocyte apoptosis. Alternatively, these observations may result from the ability of thapsigargin to elevate $[Ca^{2+}]_i$ via two independent mechanisms, namely mobilisation of Ca^{2+} from intracellular stores and an increase in the influx of extracellular Ca^{2+}. The resulting levels of Ca^{2+} within the granulocytes may therefore be greater than the level achieved by treatment of granulocytes with A23187. Direct measurements of the extent to which Ca^{2+} is elevated by thapsigargin and A23187 in eosinophils and neutrophils would need to be undertaken to make any firm conclusions regarding this hypothesis. Particularly germane to our findings, Zhu & Loh (1995) have demonstrated that mobilisation of Ca^{2+} from intracellular pools, rather than an increase in $[Ca^{2+}]_i$ alone, provides the signal for the initiation of apoptosis in HL-60 cells. This study demonstrates that a solitary increase in $[Ca^{2+}]_i$ did not result in apoptosis, while the depletion of thapsigargin-sensitive Ca^{2+} pools in the endoplasmic reticulum was closely associated with the induction of apoptosis in HL-60 cells. Moreover, they reported that the extracellular and intracellular Ca^{2+} chelators, EGTA and BAPTA, promoted apoptosis, in contrast to the calmodulin antagonist, W-13 which did not attenuate apoptosis in HL-60 cells. Collectively these findings, in conjuction with the data presented in figure 4.2, may support the interpretation that mobilisation of intracellular Ca^{2+} stores is particularly important in regulation of the apoptotic programme in myeloid cells.

Abundant evidence has emerged supporting the involvement of Ca^{2+} in apoptosis, with documented findings particularly focussing on the role of Ca^{2+} in the regulation of thymocyte apoptosis. One such report from Wyllie et al., (1984) has demonstrated that Ca^{2+} ionophores cause endonuclease activation as well as many of the morphological changes typically associated with apoptosis in thymocytes. Our results are particularly intriguing in that elevation of $[Ca^{2+}]_i$ differentially affects granulocyte apoptosis; again indicating that these cells are regulated differently. Moreover, it has been suggested that the existence of endonucleases with different requirements of Ca^{2+} for their activation may be responsible for the heterogeneous action of Ca^{2+} in the regulation of apoptosis in various cell types (Zhu & Loh, 1995). We have also investigated the role of Ca^{2+} in dexamethasone-mediated granulocyte apoptosis. Interpretation of experiments in which the Ca^{2+} elevating agents A23187 and thapsigargin were co-cultured in the presence of dexamethasone with either eosinophils or neutrophils has proven to be difficult, given that these agents individually modulate the rate of granulocyte apoptosis *in vitro* (figure 4.3). However, these experiments may suggest that elevation of $[Ca^{2+}]_i$ modulates dexamethasone-mediated granulocyte apoptosis. Significantly, further studies investigating the role of Ca^{2+} in dexamethasone-mediated inhibition of neutrophil apoptosis argue against a role for Ca^{2+}, since the anti-apoptotic effect of dexamethasone was unaffected by chelation of intracellular Ca^{2+} (figure 4.4). Furthermore, preliminary studies involving the direct measurement of cytosolic Ca^{2+} levels in freshly isolated neutrophils, did not identify any changes in the $[Ca^{2+}]_i$ above resting values of 100 nM, upon dexamethasone addition. Although our findings argue against a role for Ca^{2+} in dexamethasone-mediated inhibition of

neutrophil apoptosis, more intensive experiments are necessary to make any firm conclusions. Interestingly, contradictory results have been reported on the question of the role of Ca^{2+} in glucocorticoid-induced apoptosis in thymocytes. Early studies on the biochemical mechanism of glucocorticoid-induced apoptosis in rat thymocytes have highlighted the dependence of this induction of apoptosis on a sustained increase in $[Ca^{2+}]_i$ (Kaiser & Edelman, 1977, McConkey et al., 1989a), being inhibited by the depletion of intracellular Ca^{2+} with EGTA (Kaiser & Edelman, 1977, McConkey et al., 1989a) and buffering of intracellular Ca^{2+} with quin-2/AM (McConkey et al., 1989a). On the other hand, Iseki et al., (1993) have reported that glucocorticoid-induced apoptosis in murine and rat thymocytes is not dependent on an increase in $[Ca^{2+}]_i$. Moreover, this latter report suggests that the discrepancy between these results may be accounted for by the different methods used for measurement of $[Ca^{2+}]_i$ levels. McConkey and colleagues measured $[Ca^{2+}]_i$ using a fluorescence spectrophotometer and a cell suspension in a curvette (McConkey et al., 1989a). This classical method would give a false increase in $[Ca^{2+}]_i$ if the fluorescent Ca^{2+} indicator, fura-2, leaked from the cells by for example, cytolysis during the measurement. However, Iseki et al., (1993) assessed dexamethasone-induced $[Ca^{2+}]_i$ elevations in murine thymocytes, by microscopic fluorimetry with constant perfusion to remove leaked indicator. Nevertheless, this differing methodology in the measurement of $[Ca^{2+}]_i$ does not account for their findings that depletion of extracellular Ca^{2+} with EGTA or buffering intracellular Ca^{2+} with quin-2/AM did not inhibit glucocorticoid-induced thymocyte apoptosis in contrast to findings by McConkey and colleagues. In other systems, such as human CEM lymphocytes (a human lymphoid line of T cell derivation) glucocorticoid-induced apoptosis is induced by a Ca^{2+}-independent mechanism

(Alnemri & Litwack, 1990, Bansal et al., 1990). The involvement of Ca^{2+} in glucocorticoid-mediated thymocyte apoptosis therefore remains a complex and controversial issue.

With increasing evidence implicating a role for Ca^{2+} in the regulation of apoptosis, an important aspect of ongoing research involves defining the targets of Ca^{2+} in apoptotic cells. Elevation of $[Ca^{2+}]_i$ may regulate apoptosis by direct effects on the enzymatic activities of proteases and/or endonucleases responsible for mediating cellular demise in apoptosis. Squier and colleagues have recently demonstrated that activation of the Ca^{2+}-dependent neutral protease calpain is involved in glucocorticoid- and irradiation-induced thymocyte apoptosis (Squier et al., 1994, Squier & Cohen, 1997). Moreover, other Ca^{2+}-sensitive proteases may also participate in the apoptotic process. Previous work has demonstrated that a family of nuclear matrix proteins, the lamins are degraded in cells undergoing apoptosis (Neamati et al., 1995, Oberhammer et al., 1994) and in thymocytes lamin cleavage occurs via a Ca^{2+}-dependent mechanism (Neamati et al., 1995). Alternatively, Ca^{2+} may regulate apoptosis via mechanisms involving activation of Ca^{2+}-dependent protein kinases and/or phosphatases leading to alterations in gene transcription. Studies with the immunosuppressive drugs cyclosporin A (CsA) and FK506 support this model for how Ca^{2+} elevation may be coupled to downstream events in apoptosis. CsA and FK506 bind to a family of cytosolic receptors promoting direct binding to and inhibition of the Ca^{2+}/calmodulin-dependent protein serine/threonine phosphatase, calcineurin (PP2B) (Liu et al., 1991). Evidence has now been presented from several laboratories illustrating that CsA can block Ca^{2+}-dependent apoptosis in lymphoid model systems (Shi et al., 1989, Amendola et al.,

1994, Bonnefoy-Berard et al., 1994, Makrigiannis et al., 1994), indicating that calcineurin activation may be required for these responses and thereby suggesting that calcineurin is one target for Ca^{2+} in apoptosis. However, not all pathways of apoptosis are affected by these immunosuppressive agents. Recent work confirms that CsA and FK506 block Ca^{2+}-stimulated apoptosis in T cell hybridomas but are without effect on Ca^{2+}-dependent apoptosis in immature rodent thymocytes (McConkey & Orrenius, 1996). Collectively, these findings suggest that while calcineurin may represent a target for Ca^{2+} in apoptosis, it is not a universal target. Recently, Dolmetsch et al., (1997) have shown that the amplitude and duration of Ca^{2+} signals in B lymphocytes controls differential activation of the pro-inflammatory transcriptional regulators NF-*k*B, JNK and NFAT. They report that NF-*k*B and JNK are selectively activated by a large transient $[Ca^{2+}]_i$ rise, whereas NFAT is activated by a low, sustained Ca^{2+} plateau. These results show how downstream effectors can decode information in relation to the amplitude and duration of Ca^{2+} signals, revealing a mechanism by which Ca^{2+} can achieve specificity in signalling to the nucleus. These recent observations may therefore also prove to be relevant in the mechanism by which Ca^{2+} regulates apoptosis and mediates differential cell signalling.

We have also demonstrated that treatment of both eosinophils and neutrophils with inhibitors of PKC induces apoptosis (figures 4.6 and 4.7). Moreover, the pro-apoptotic effect upon neutrophils was noted in response to both a relatively specific PKC inhibitor, Ro-31-8220 (Keller & Niggli, 1993), as well as by the relatively nonspecific inhibitor, staurosporine (Schachtele et al., 1988, Nakadate et al., 1988). Collectively, these data implicate a role for PKC in the suppression of granulocyte

apoptosis. Based upon our findings and those of several other laboratories, the role of PKC in apoptotic events appears to vary with cell type. In certain cells, phorbol ester-stimulated activation of PKC inhibits apoptosis while in other cells a death programme is initiated by activation of PKC (McConkey et al., 1989d, Araki et al., 1990, Forbes et al., 1992, Sun et al., 1992, Ishii & Gobe, 1993). Activation of the PKC signal transduction pathway appears to modulate apoptosis in a cell-specific fashion. However, apart from describing the ability of PKC to modulate apoptosis, very little is known with regards to the mechanisms responsible for producing these divergent, cell-specific responses. It has now been established that PKC is not a single molecular entity but infact exists as many closely related PKC isotypes. The existence of at least twelve members of the PKC gene family, indicated by the Greek symbols α, βI, βII, γ, δ, ε, ξ, η, θ, ι, λ and μ, may serve as a mechanism responsible for the differential regulation by PKC of apoptosis (Dekker & Parker, 1994). It is likely that a full range of PKC isotypes has not yet been identified and that even more members of the PKC family will be discovered in the future. Differences in the expression patterns, substrate specificity, subcellular location and sensitivity to agonists among the individual PKC isoforms may serve as possible mechanisms by which apoptosis is modulated by PKC in a cell-specific manner. The expression pattern of PKC isoenzymes in different tissues and cell lines is now emerging with isotypes β1, βII, δ, ξ and η being ubiquitously expressed in myeloid cell lines (Hug & Sarre, 1993).

The existence of different types of PKC with distinct properties may explain the apparent contradiction of results between the role of PKC activation in inducing thymocyte apoptosis as reported by both Kizaki et

al., (1989a) and Ojeda et al., (1992), with that of McConkey et al., (1989d), who reported inhibition of DNA fragmentation with agents that stimulate PKC.

We have also demonstrated that staurosporine and Ro-31-8220, at a concentration (0.1 μM) that did not modulate the rate of constitutive neutrophil apoptosis, partially abrogated dexamethasone-mediated inhibition of neutrophil apoptosis. These data suggest that the mechanism underlying dexamethasone-mediated inhibition of neutrophil apoptosis may involve activation of PKC. The documented effects of PKC in the regulation of glucocorticoid-mediated apoptosis, in other cells, again appears to be cell-type specific. Ojeda and colleagues have demonstrated that inhibition of PKC by exposure to H7 prevents glucocorticoid-induced apoptosis in murine thymocytes, suggesting that activation of PKC promotes this process (Ojeda et al., 1990). Whereas, McConkey et al., (1989d) have shown that activation of PKC using PMA opposes steroid-induced apoptosis in thymic lymphocytes, consistent with an antagonistic influence of PKC. Moreover, Iwata et al., (1994) have reported that glucocorticoid-induced apoptosis in immature thymocytes involves glucocorticoid receptor-mediated and selective activation of PKC-ε, through *de novo* synthesis of macromolecules. We have previously demonstrated that granulocyte apoptosis is dependent on protein synthesis (Chapter 3), and as such glucocorticoids may activate unknown genes involved in the production of a protein which induces activation of one or more PKC isoenzymes. However, at present the role of PKC in the regulation of glucocorticoid-mediated granulocyte apoptosis and the mechanism of action of PKC remain unclear.

With the advent of isotype-specific PKC stimulators and inhibitors, it is now becoming possible to assess the specific role of PKC isoenzymes in the regulation of apoptosis. Recent reports have indicated that PKC isoenzymes are differentially involved in the regulation of apoptosis. The potential importance of changes in PKC-β expression during apoptosis has been suggested by several laboratories. Knox and colleagues have demonstrated that the level of bcl-2, a protein known to prevent apoptosis in many cell types, was inversely related to the expression of PKC-β and -α in tonsil epithelial cells (Knox et al., 1993). Moreover, a phorbol ester capable of selectively activating PKC-βI *in vitro* has been shown to accelerate apoptosis in U937 cells (Pongracz et al., 1994) and HL60 cells (Macfarlane & Manzel, 1994). It has therefore been suggested that these findings may collectively indicate that PKC-β is an important element regulating myeloid cell apoptosis. Furthermore, Pongracz et al., (1995) have more recently reported that the level of certain PKC isoenzymes changed during spontaneous apoptosis of U937 promonocytic cells; with an increase in PKC-β and a decrease in PKC-ξ. Moreover, the intracellular location of PKC-α appeared to be altered in apoptotic U937 cells, being associated more with the nucleus than the cytosol. These data suggest that the levels and disposition of the PKC isoenzymes are modulated during apoptosis. Finally, Emoto et al., (1995) have reported that apoptosis in irradiated U937 cells is associated with proteolytic activation of PKC-ξ by an ICE-like protease since the specific tetrapeptide ICE inhibitor (YZAD) blocked both proteolytic activation of PKC-ξ and internucleosomal DNA fragmentation. These findings indicate that PKC-ξ is a downstream target of the ICE-like protease and may provide clues to the potential targets of PKC-mediated myeloid cell apoptosis.

The connection between PKC activation and modulation of apoptosis is presumably mediated by proteins, which become phosphorylated by PKC (Azzi et al., 1992). One such PKC substrate is I*k*B, a 35 kDa protein which is able to bind to and inhibit the transcription factor NF-*k*B. The inactive complex I*k*B/NF-*k*B is phosphorylated by PKC on the inhibitor protein (Ghosh & Baltimore, 1990), allowing NF-*k*B to move to the nucleus where it exerts specific DNA binding ability and is involved in the activation of target genes (Finco & Baldwin, 1995). Moreover, it has been recently reported that glucocorticoids inhibit NF-*k*B activity, specifically by increasing synthesis of I*k*B (Didonato et al., 1996, Barnes & Adcock, 1997). Thus the mechanism underlying PKC directed constitutive and glucocorticoid-mediated apoptosis, may involve target proteins such as I*k*B.

In summary, our present studies provide evidence that elevation of $[Ca^{2+}]_i$ differentially affects the rate of granulocyte apoptosis; promoting eosinophil apoptosis and inhibiting neutrophil apoptosis. Moreover, exposure of these granulocytes to PKC inhibitors of varying specificities triggers apoptosis, which indirectly suggests that the basal activity of one or more isoforms of PKC protects these cells from apoptosis. Thus our findings implicate both Ca^{2+} and PKC signal transduction pathways in the regulation of granulocyte apoptosis. However, further studies are necessary to fully elucidate the mechanisms by which Ca^{2+} and PKC either independently or synergistically modulate granulocyte apoptosis.

Chapter 5

The role of MAPK/ERK and protein phosphatases in basal and glucocorticoid-mediated granulocyte apoptosis

5.1 Introduction

The level of protein phosphorylation in any cell ultimately depends upon the integrated activities of both protein kinases and protein phosphatases. The resulting phosphorylation and dephosphorylation of various substrate proteins is a principle mechanism of signal transduction in eukaryotic cells and is becoming recognised as being essential in the control of cellular survival.

Mitogen-activated protein kinases (MAPKs) are important mediators of signal transduction from the cell surface to the nucleus. Regulation by MAPKs has been implicated in the mediation of a wide array of physiological processes including cell shape, osmotic integrity and pheromone responses in yeasts (Ammerer, 1994, Herskowitz, 1995), stress responses in mammalian cells (Galcheva-Gargova et al., 1994, Han et al., 1994, Rouse et al., 1994), cytokine signalling (Freshney et al., 1994) and cell growth and proliferation (Davis, 1993). However, the involvement of MAP kinases in the regulation of apoptosis has not been studied in any depth.

There is good evidence that MAP kinase plays a key role in the transduction of signals through both protein kinases and protein phosphatases. The specific regulation of protein phosphatases indicates their central role in cellular regulation and the discovery of many cell

permeable phosphatase inhibitors, such as okadaic acid and calyculin A, has assisted the study of the function of protein phosphatases in cellular regulation (Holmes & Boland, 1993).

Although their precise functions are not yet known, several biological roles of protein phophatases have been revealed in a number of recent studies (Axton et al., 1990, Kinoshita et al., 1990, Nitschke et al., 1992). PP1 (and PP2A) have been implicated in many different cellular processes, as diverse as glycogen metabolism, calcium transport, muscle contraction, protein synthesis and intracellular transport (Cohen, 1989, Shenolikar & Nairn, 1991, Bollen & Stalmans, 1992). Moreover, the protein phosphatase inhibitors, okadaic acid and calyculin A, have been shown to modify several neutrophil responses such as altered shape change and F-actin distribution, suggesting that dephosphorylation has a regulatory role in myeloid cells (Kreienbuhl et al., 1992). Phosphatase type 1 appears to be a major regulator of the cAMP-response element binding protein (CREB) (Hagiwara et al., 1992) and it has been suggested that these phosphatases may regulate the activity of multiple transcription factors (Park et al., 1992). Other studies have provided evidence that dephosphorylation of proteins may play a role in both the positive and negative regulation of apoptosis. Both okadaic acid and calyculin A have been shown to protect cells against apoptotic cell death induced by heat and ionizing radiation in tumour cell lines (Baxter & Lavin, 1992), Burkitt's lymphoma cell line BM13674 cells (Song & Lavin, 1993) and from apoptosis induced by a number of cytotoxic compounds (Song et al., 1992). Conversely, okadaic acid has been shown to induce apoptosis in leukemic cells (Bøe et al., 1991, Ishida et al., 1992).

In this study we have used PD 098059, an inhibitor of the MAPK/ERK cascade and both okadaic acid and calyculin A, protein phosphatase inhibitors, to investigate the potential involvement of MAP kinase and PP1 and PP2A, in basal and glucocorticoid-mediated eosinophil and neutrophil apoptosis.

5.2 Results

5.2.1 Involvement of MAP kinase in eosinophil and neutrophil apoptosis

Eosinophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM showed an inhibition in the light microscopic features of apoptosis in the presence of the MAP kinase cascade inhibitor, PD 098059 (50 μM) (Figure 5.1*A*). In contrast to the effects observed on eosinophils, PD 098059 (50 μM) had no significant effect upon the rate of apoptosis in neutrophils ($2x10^6$/ml), cultured in serum-supplemented Iscove's DMEM (Figure 5.1*B*). Treatment of both cell types with PD 098059 did not significantly alter either cell viability or recovery. These results illustrate that the basal rates of eosinophil and neutrophil apoptosis are regulated differently. Inhibition of the MAP kinase signalling cascade appears to inhibit eosinophil apoptosis, while having no effect on the rate of neutrophil apoptosis.

5.2.2 Involvement of MAP kinase in glucocorticoid-mediated eosinophil and neutrophil apoptosis

We subsequently investigated the effect of PD 098059 on the rate of glucocorticoid-mediated granulocyte apoptosis. Figure 5.2 illustrates that eosinophils pre-incubated for 30 min with PD 098059 (50 μM), before addition of dexamethasone (1 μM), showed a slight inhibition of the pro-apoptotic effect observed in the presence of dexamethasone alone. Moreover, the extent of this inhibition was comparable to the inhibitory effect of PD 098059 on the basal rate of eosinophil apoptosis. Thus, it is difficult to draw mechanistic conclusions from such an experiment,

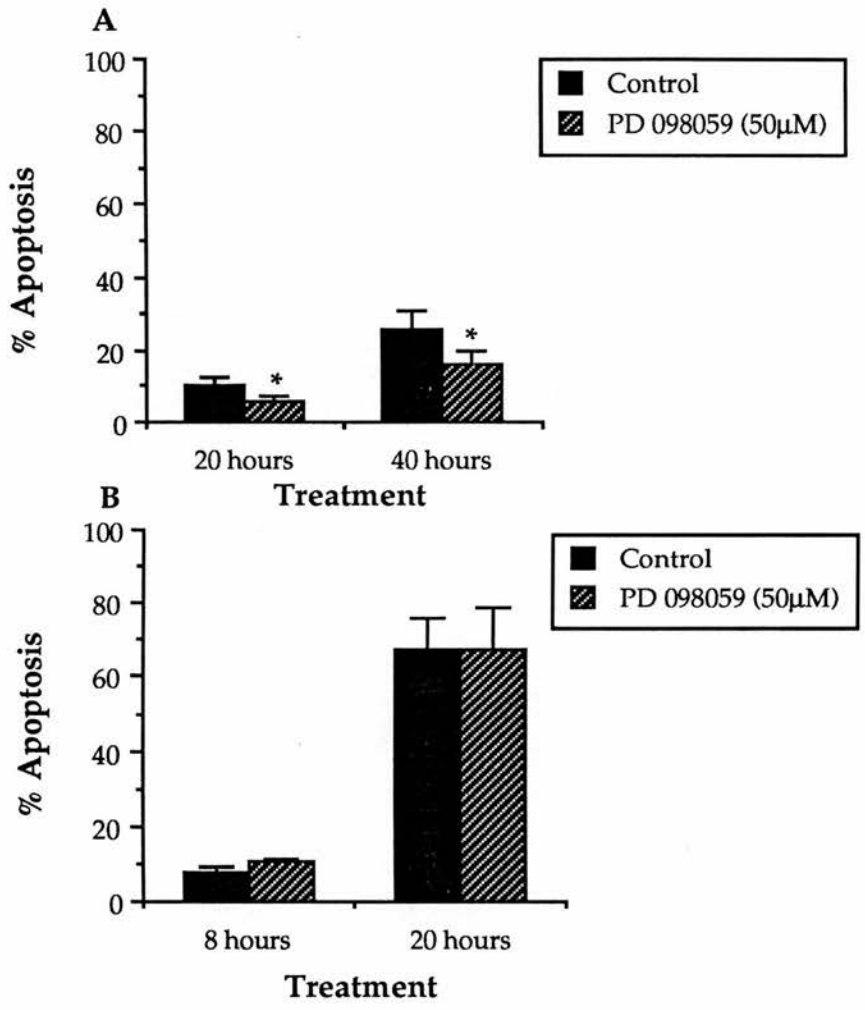

Figure 5.1 Effect of PD 098059 on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured for 20 and 40 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of PD 098059 (50 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 6 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured for 8 and 20 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of PD 098059 (50 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

given that PD 098059 *per se* modulates the rate of basal eosinophil apoptosis in culture. We were also interested in the effect of this MAP kinase cascade inhibitor on other inflammatory mediators known to inhibit eosinophil apoptosis. Figure 5.2 illustrates that PD 098059 (50 μM) does not significantly modulate IL-5- or GM-CSF-mediated inhibition of eosinophil apoptosis.

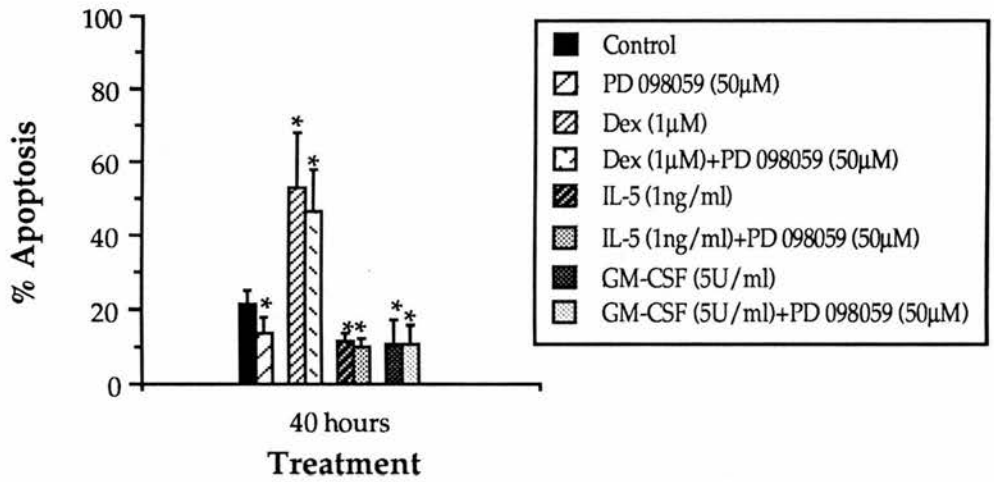

Figure 5.2 Effect of PD 098059 on dexamethasone-, IL-5- and GM-CSF-mediated eosinophil apoptosis.
Eosinophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), IL-5 (1 ng/ml) or GM-CSF (5 U/ml), in the presence and absence of PD 098059 (50 μM). Eosinophils were pre-incubated with PD 098059 for 30 min at 37°C in each case. Cells were harvested following 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-5 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

The effect of PD 098059 on dexamethasone-, LPS-, dibutyryl-cAMP, and GM-CSF-mediated neutrophil apoptosis is illustrated in figure 5.3. Interestingly, PD 098059 while having no effect on the basal rate of

neutrophil apoptosis, or dexamethasone-, dibutyryl-cAMP- and GM-CSF-mediated apoptosis, completely attenuated LPS-mediated inhibition of neutrophil apoptosis. These data suggest that glucocorticoid-induced inhibition of neutrophil apoptosis is not mediated by the MAPK/ERK cascade. However, LPS-induced inhibition of neutrophil apoptosis may involve activation of the MAPK/ERK cascade.

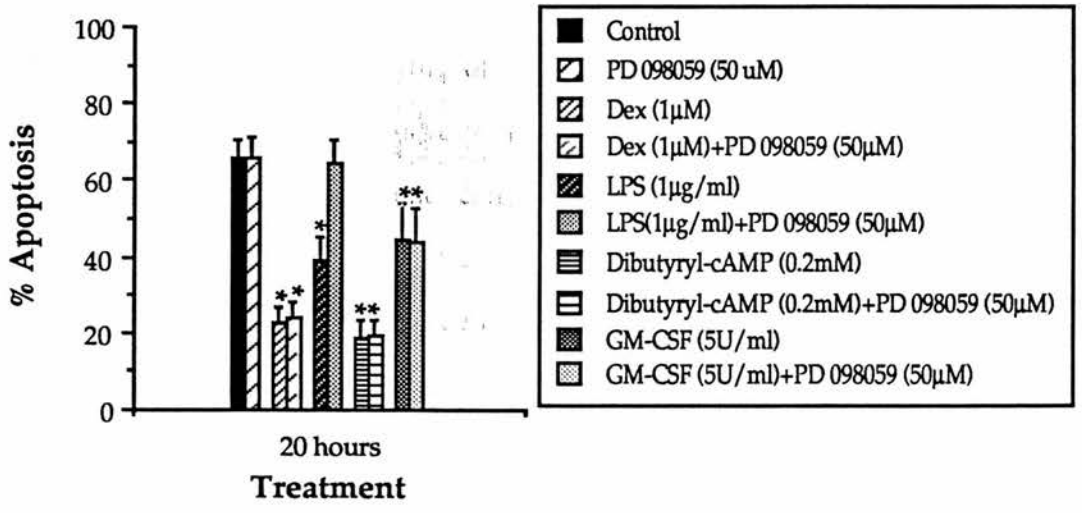

Figure 5.3 Effect of PD 098059 on dexamethasone-, LPS-, dibutyryl-cAMP-, and GM-CSF-mediated neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), LPS (1 μg/ml), dibutyryl-cAMP (0.2 mM) or GM-CSF (5 U/ml), in the presence and absence of PD 098059 (50 μM). Neutrophils were pre-incubated with PD 098059 for 30 min at 37°C in each case. Cells were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 5-8 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

5.2.3 Involvement of protein phosphatases in basal and glucocorticoid-mediated granulocyte apoptosis

In order to gain a better understanding of the role of protein phosphorylation in the regulation of basal and glucocorticoid-mediated granulocyte apoptosis, we investigated the effects of the protein phosphatase inhibitors, okadaic acid and calyculin A. Figure 5.4*A* illustrates the effect of okadaic acid (0.5-100 nM) on the rate of eosinophil apoptosis, after 20 and 40 h in culture. At the time periods studied, the lower concentrations of okadaic acid ($\leq$ 5 nM) caused a decrease in the rate of eosinophil apoptosis, while the higher concentrations (> 50 nM) promoted apoptosis, as compared to the control population of cells. Figure 5.4*B* illustrates the effect of okadaic acid (0.5-500 nM) on the rate of neutrophil apoptosis, after 8 and 20 h in culture. At the time periods studied, the lower concentrations of okadaic acid ($\leq$ 50 nM) caused a decrease in the rate of neutrophil apoptosis, while the higher concentrations (> 100 nM) promoted apoptosis, as compared to the control population of cells. These results indicate that okadaic acid has a bi-phasic effect on the rate of both eosinophil and neutrophil apoptosis, with the lower concentrations inhibiting and the higher concentrations promoting apoptosis, without inducing cellular necrosis at the concentrations shown. The range of concentrations of okadaic acid used, however, varied between the two cell types. A high proportion of eosinophils (>80%) admitted trypan blue when cultured with concentrations of okadaic acid > 100 nM, suggesting that this concentration of okadaic acid was toxic to the cell and therefore induced cell necrosis. In contrast, okadaic acid (0.5-500 nM) did not induce cellular necrosis in neutrophils with the viability reproducibly being > 90% at the time periods indicated

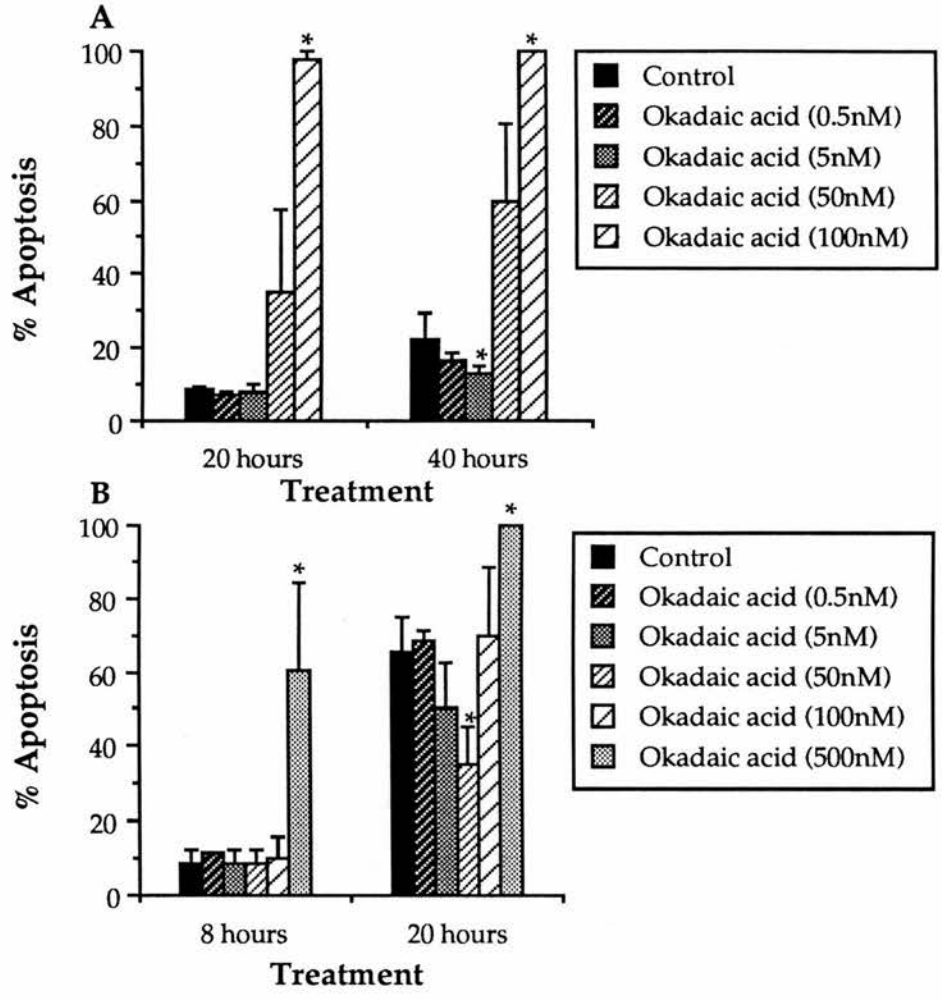

Figure 5.4 Effect of okadaic acid on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured for 20 and 40 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of okadaic acid (0.5-100 nM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured for 8 and 20 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of okadaic acid (0.5-500 nM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

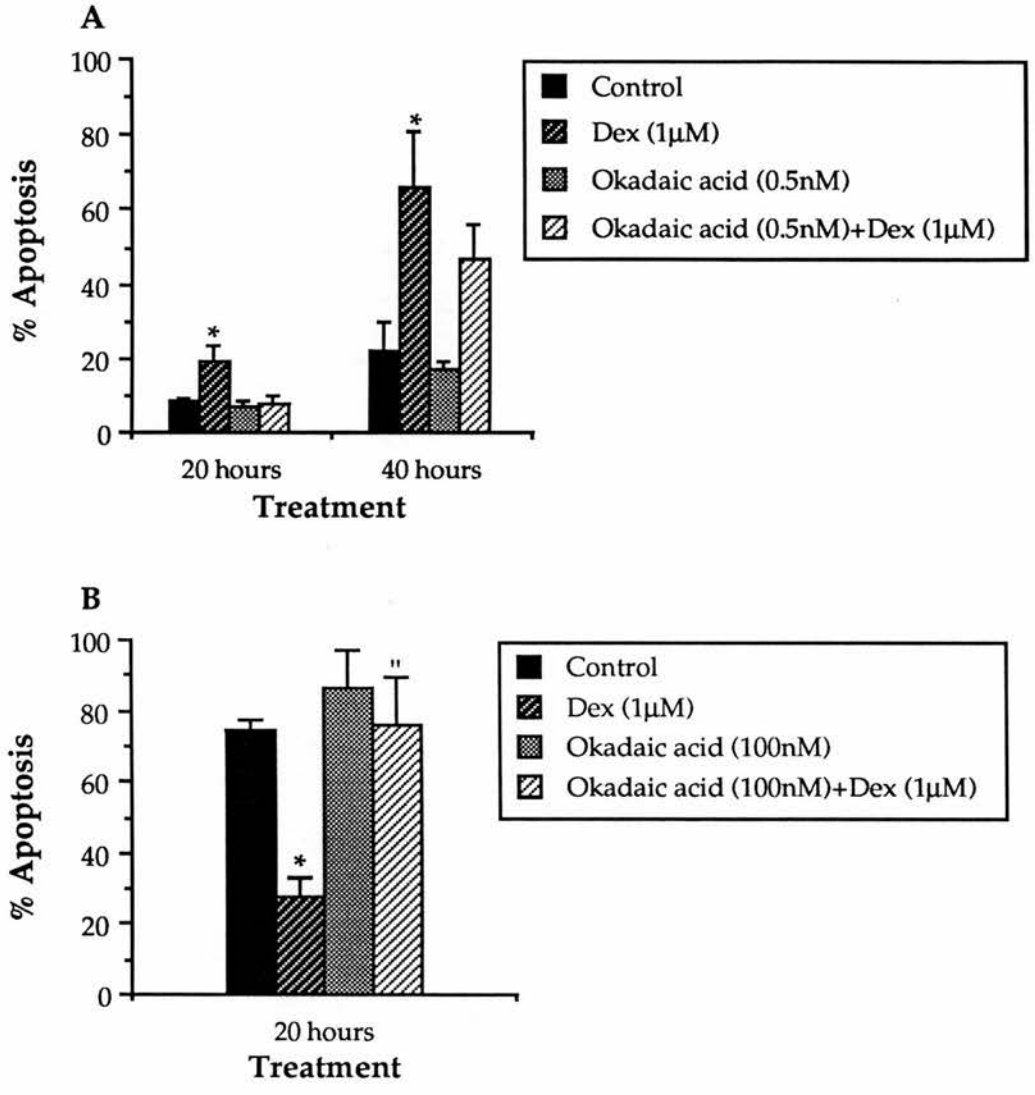

Figure 5.5 Effect of okadaic acid on dexamethasone-mediated eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of okadaic acid (0.5 nM). Eosinophils were harvested following 20 and 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of okadaic acid (100 nM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). Data obtained for okadaic acid plus dexamethasone varied significantly from dexamethasone alone (" $p<0.05$).

in figure 5.4*B*. Eosinophils therefore appear to be more sensitive to the cytotoxic effects of okadaic acid than neutrophils.

Figure 5.5 illustrates the effect of okadaic acid on glucocorticoid-mediated granulocyte apoptosis. Okadaic acid (0.5 nM) had no significant effect upon the control rate of eosinophil apoptosis while partially attenuating the pro-apoptotic effect of dexamethasone (Figure 5.5*A*). This okadaic acid-induced partial attenuation of dexamethasone-mediated eosinophil apoptosis was consistently observed in each individual experiment. However, further experiments are required since the significance of these results is masked by donor variation between the basal levels of eosinophil apoptosis. Nevertheless, dexamethasone-mediated promotion of eosinophil apoptosis is significantly different from the control population of cells and co-culture of okadaic acid (0.5 nM) and dexamethasone (1 μM) renders the dexamethasone effect to be no longer significant. Figure 5.5*B* illustrates that okadaic acid (100 nM) had no significant effect upon the control rate of neutrophil apoptosis while completely attenuating dexamethasone-mediated inhibition of neutrophil apoptosis. These data suggest that modulation of cellular phosphatases may be an important factor in the regulation of glucocorticoid-mediated granulocyte apoptosis.

An alternative protein phosphatase inhibitor, calyculin A, was therefore used to assess the reproducibility of these results on neutrophils. Figure 5.6*A* illustrates that calyculin A (1-100 nM) has no significant effect on the basal rate of neutrophil apoptosis after 20 h in culture. Moreover, calyculin A (1-100 nM) induced a partial attenuation of dexamethasone-mediated inhibition of neutrophil apoptosis (figure 5.6*B*). As illustrated

in figure 5.6*B*, dexamethasone-mediated inhibition of neutrophil apoptosis is significantly different from the control population of cells and co-culture of calyculin A (1-100 nM) and dexamethasone (1 μM) renders the dexamethasone effect to be no longer significant. These data strengthen the hypothesis that modulation of protein phosphatases may play a role in the regulation of glucocorticoid-mediated neutrophil apoptosis.

Finally, to investigate the specificity of these results, we compared the effect of calyculin A on dexamethasone-, LPS-, dibutyryl-cAMP-, and GM-CSF-mediated neutrophil apoptosis (figure 5.7). Interestingly, calyculin A while having no effect on the basal rate of neutrophil apoptosis, also failed to modulate LPS-, dibutyryl-cAMP-, and GM-CSF-mediated inhibition of neutrophil apoptosis. These data suggest that the partial attenuation of dexamethasone-mediated inhibition of neutrophil apoptosis by calyculin A, is specific to dexamethasone, since calyculin A does not modulate the inhibition of neutrophil apoptosis mediated by LPS, dibutyryl-cAMP or GM-CSF.

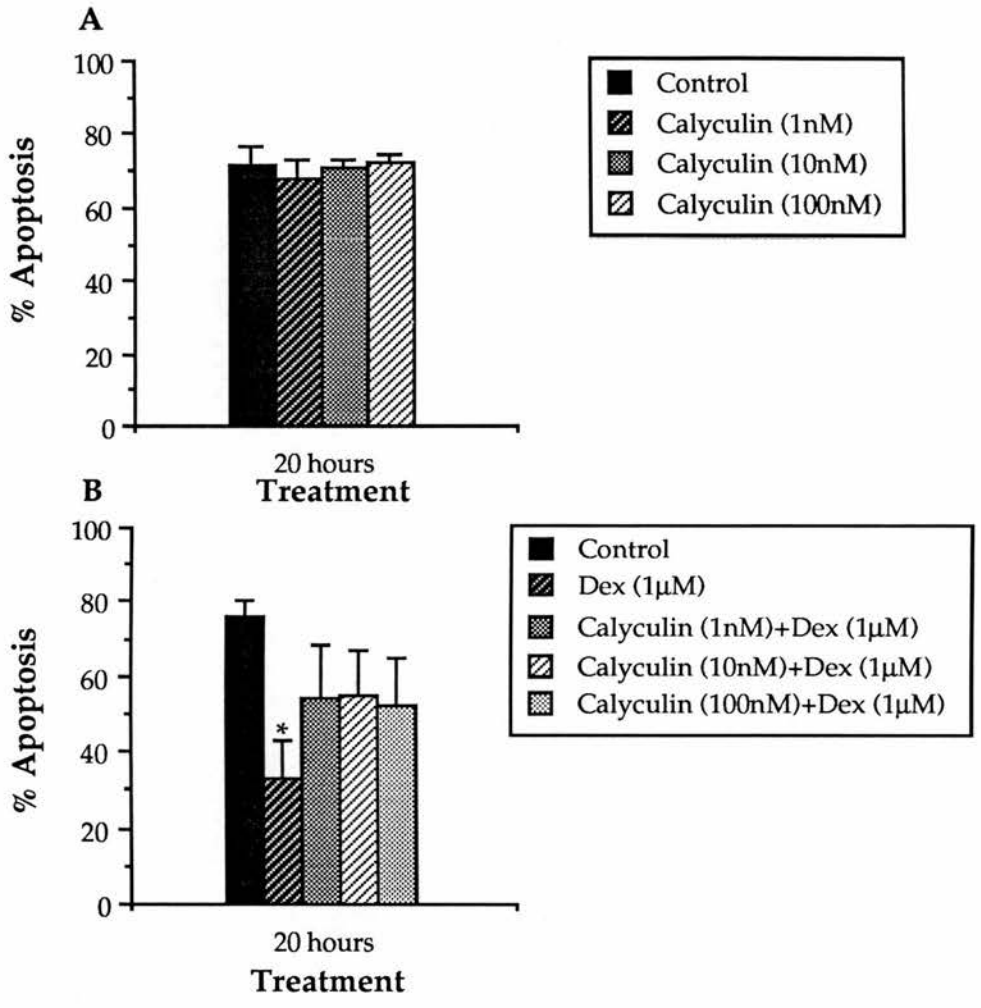

Figure 5.6 Effect of calyculin on basal and dexamethasone-mediated inhibition of neutrophil apoptosis.

A, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of calyculin (1-100 nM). Cells were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of calyculin (1-100 nM). Cells were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

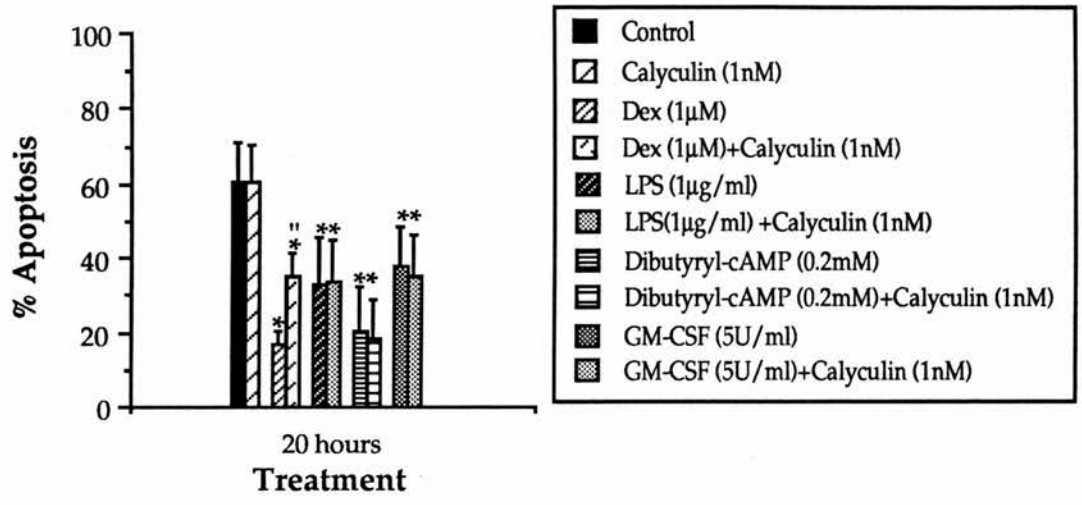

Figure 5.7 Effect of calyculin on dexamethasone-, LPS-, dibutyryl-cAMP, and GM-CSF-mediated neutrophil apoptosis.

Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), LPS (1 μg/ml), dibutyryl-cAMP (0.2 mM) or GM-CSF (5 U/ml), in the presence and absence of calyculin (1 nM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). Data obtained for calyculin plus dexamethasone varied significantly from dexamethasone alone (" $p<0.05$).

5.3 Discussion

We have demonstrated that PD 098059, a synthetic inhibitor of the MAPK/ERK pathway (Alessi et al., 1995, Dudley et al., 1995), down-regulates the basal rate of eosinophil apoptosis as compared to the control population of cells (figure 5.1*A*), while having no effect on the basal rate of neutrophil apoptosis (figure 5.1*B*). These data indicate that activation of the MAPK/ERK signalling cascade may be an important factor in driving eosinophils through the apoptotic process and may offer a potential mechanism underlying the promotion of eosinophil apoptosis, thereby contributing to the resolution of inflammation. PD 098059 is reportedly a selective inhibitor of the MAPK-activating enzyme MEK, and moreover appears to be relatively specific towards inhibition of MEK 1 as opposed to MEK 2 activation (Alessi et al., 1995). Our data may therefore implicate the specific involvement of $p44^{MAPK}$ (ERK 1) in the regulation of eosinophil apoptosis. In view of these findings, we investigated the effect of PD 098059 on dexamethasone-mediated promotion of eosinophil apoptosis. Moreover, inflammatory mediators such as IL-5 and GM-CSF, known to inhibit eosinophil apoptosis (Stern et al., 1992), were also studied. PD 098059 was shown to marginally inhibit dexamethasone-mediated eosinophil apoptosis in a manner comparable to the inhibitory effect of PD 098059 observed upon the basal rate of eosinophil apoptosis. Moreover, PD 098059 did not significantly modulate IL-5- or GM-CSF-mediated inhibition of eosinophil apoptosis. However, although these data highlight a difference between the effect of PD 098059 on dexamethasone-mediated compared with IL-5- and GM-CSF-mediated eosinophil apoptosis, it is difficult to draw mechanistic conclusions from such experiments, given that PD 098059 alone modulates the rate of basal eosinophil apoptosis in culture. Clearly, further investigations are

needed, particularly in light of reports that the MAPK cascade is activated in response to a variety of cytokines (Feig, 1993), although the mechanisms involved have not been fully elucidated. Interest has particularly focussed around a possible link between the β-subunit of IL-3/IL-5/GM-CSF receptors, which is the subunit that has been shown to be the common signal transducer for these cytokines, and activation of the Ras pathway (Sato et al., 1993). Moreover, Yousefi et al., (1997) have highlighted that in the absence of an intrinsic tyrosine kinase domain in the β-subunit, activation of the Ras pathway may involve receptor-associated tyrosine kinases such as Lyn or Syk, which are src-type tyrosine kinases. This hypothesis is strengthened by the finding that these tyrosine kinases have been shown to be essential for IL-3/IL-5/GM-CSF-mediated inhibition of eosinophil apoptosis (Yousefi et al., 1996). Furthermore, these authors have also reported that since Jak2 constitutively associates with the IL-3/IL-5/GM-CSF receptor β-subunit in human eosinophils, Jaks may also be involved in the activation of the Ras pathway (Yousefi et al., 1997). Thus, this report speculates that these kinases either collectively or individually may activate the Ras pathway to transcribe anti-apoptotic genes in eosinophils, thereby inferring that activation of the MAP kinase signalling cascade in eosinophils suppresses apoptosis. However, our results presented in figure 5.1*A* suggest that activation of the MAP kinase pathway promotes eosinophil apoptosis. Clearly, further experiments are required to determine conclusively, the role of MAPK in the regulation of eosinophil apoptosis and to fully elucidate the mechanism by which inflammatory cytokines such as IL-5 inhibit eosinophil apoptosis.

In contrast to the results observed in eosinophils, PD 098059 while having no effect on the basal rate of neutrophil apoptosis or dexamethasone-, dibutyryl-cAMP- and GM-CSF-mediated apoptosis, completely attenuated LPS-mediated inhibition of neutrophil apoptosis (figure 5.3). Moreover, Paul and colleagues have also demonstrated that in human neutrophils, where LPS delays the constitutive rate of apoptosis, PD 098059 (a MAPK/ERK cascade inhibitor) completely suppressed and SB 203580 (a p38 MAP kinase inhibitor) partially suppressed the effect of LPS (Paul et al., 1997). In agreement with our findings, these results implicate MAPKs/ERKs and to a lesser extent, p38 MAP kinase, in LPS-induced cellular protection of neutrophils. Previous reports demonstrate that LPS has been shown to stimulate both the 'classical' Ras-dependent MAPK/ERK pathway and the cytokine-activated p38 MAP kinase. For example, in certain murine cell lines LPS has been reported to result in MAPK/ERK phosphorylation and activation (Weinstein et al., 1992, Dong et al., 1993). However, in neutrophils (Nahas et al., 1996, Nick et al., 1996), a murine pre-B cell line, 70Z/3, transfected with human CD14 (Han et al., 1994) and PC-12 cells (Rouse et al., 1994), LPS induces phosphorylation and activation of p38 MAP kinase, whose *in vivo* substrates include heat shock protein (hsp) 27 (Cuenda et al., 1995) and the transcription factor, CREB (Tan et al., 1996). Interestingly, the p90 ribosomal S6 kinase, a downstream target of MAPKs/ERKs, is also able to phosphorylate CREB at Ser-133, enabling stimulation of gene transcription (Ginty et al., 1994). It is therefore possible that phosphorylation of CREB by p90 ribosomal S6 kinase may be an important factor regulating LPS-induced inhibition of neutrophil apoptosis in our system.

The importance of the classical MAP kinases in protection against apoptosis is consistent with recent findings in PC-12 cells, where apoptosis is suppressed by NGF, which activates the MAPKs/ERKs (Xia et al., 1995). In these studies, withdrawal of NGF from the culture medium led to the sustained activation of JNK and p38 MAP kinases and inhibition of ERKs, which was accompanied by a stimulation of apoptosis. These results are particularly interesting as they indicate that apoptosis in NGF-differentiated PC-12 cells is regulated by the opposing actions of ERK and JNK-p38 MAP kinase pathways. As suggested by Xia and colleagues, the dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways may be an important factor determining whether a cell survives or undergoes apoptosis.

We have also demonstrated that PD 098059 did not modulate dexamethasone-, dibutyryl-cAMP- or GM-CSF-mediated inhibition of neutrophil apoptosis (figure 5.3). Moreover, these are the first results to our knowledge, indicating that MAPKs/ERKs are not involved in the cellular protection of neutrophils mediated by these agents. However, GM-CSF has recently been shown to increase the kinase activity of both p44 and p42 MAP kinases (Waterman & Sha'afi, 1995) and to a lesser extent the p38 MAP kinase, in human neutrophils (Nahas et al., 1996). It therefore seems likely that inflammatory mediators such as LPS and GM-CSF, regulate cell activation and apoptosis differently through activation of various MAP kinase cascades. Both agents have been shown to stimulate MAPK/ERK and p38 MAP kinase to some degree. However, in the control of the apoptotic process, the regulation of MAP kinases by these agents appears to be mediated differently, with LPS utilizing both MAPK/ERK and p38 MAP kinase again to some degree, while GM-CSF

does not appear to modulate neutrophil apoptosis with the involvement of any MAP kinase.

Furthermore, although the MAPK/ERK cascade was not implicated in dibutyryl-cAMP-mediated inhibition of neutrophil apoptosis (figure 5.3), the cAMP/PKA pathway has been shown to both positively and negatively regulate MAP kinase pathways. Previous studies have demonstrated cross-talk between the Ras/Raf/MEK/MAP kinase cascade and the cAMP second messenger system at the level of Raf-1. Cyclic-AMP has been shown to inhibit the activation of Raf-1, resulting in inhibition of MAP kinase activation in a variety of cell types including Rat-1 and NIH 3T3 fibroblasts, rat adipocytes and human arterial smooth muscle cells (Burgering et al., 1993, Cook & McCormick, 1993, Graves et al., 1993, Sevetson et al., 1993, Wu et al., 1993). It has further been shown that Raf-1 is phosphorylated by PKA at a consensus site within the Raf-1 regulatory domain (Wu et al., 1993). This phosphorylation reduces the affinity with which Raf-1 binds to Ras, suggesting that it is responsible for cAMP-mediated inhibition of Raf-1. However, the phenomenon of PKA-mediated inhibition of MAP kinase activity is not universal. In the EAhy 926 endothelial cell line no acute inhibition of growth-factor stimulated MAP kinase is observed (McLees et al., 1995). Moreover, positive regulation of MAP kinases by cAMP has been shown to occur in neuronal cells, such as PC-12 cells (Frodin et al., 1994, Young et al., 1994) and in B-16 melanoma cells (Englaro et al., 1995). These findings suggest the presence of multiple Raf isoforms, which are differentially regulated by PKA or alternatively, the presence of different PKA isoenzymes with differing affinities for Raf. Erhardt et al., (1995) have exploited a major difference between the Ras/Raf/MEK/MAP kinase cascades in PC-12 cells and Rat-1

fibroblasts, which is likely to account for the differential regulation of this pathway by cAMP/PKA. These cells reportedly contain two different isotypes of Raf, Raf-1 and B-Raf, which are differentially sensitive to inhibition by cAMP. B-Raf expression can contribute to cell type-specific differences in the regulation of the MAP kinase signalling pathway, most probably due to the fact that the PKA consensus site found in the regulatory domain of Raf-1 is not present in B-Raf (Sithanandam et al., 1990). Erhardt et al., (1995) confirmed that Raf-1 was effectively inhibited by cAMP in both PC-12 and Rat-1 cells independent of growth conditions. Conversely, B-Raf was refractory to inhibition by cAMP in PC-12 cells maintained in normal growth medium, although it was inhibited by cAMP in serum-starved cells as previously reported (Peraldi et al., 1995, Vaillancourt et al., 1994). These findings illustrate that the expression of B-Raf is an important determinant of the differential responses of PC-12 and Rat-1 cells to cAMP. Moreover, this report also pointed out that while expression of B-Raf in Rat-1 cells was sufficient to make the MAP kinase pathway resistant to inhibition by cAMP, it did not make MAP kinase inducible by cAMP alone. Interestingly, stimulation of C5a, FMLP and IL-8 receptors by their respective ligands has been shown to trigger the MAPK pathway in neutrophils through Ras/Raf-mediated events (Knall et al., 1996). Knall and colleagues have therefore suggested that these observations implicating the involvement of the MAP kinase cascade may also provide a mechanism whereby host defence mechanisms are co-ordinately regulated in the neutrophil, since these chemoattractants are able to activate both neutrophil respiratory burst and degranulation responses. Moreover, cell-permeable analogs of cAMP potently but transiently inhibited Raf activation by FMLP in neutrophils (Worthen et al., 1994). These results, when considered in conjunction

with the many studies linking cAMP to inhibition of neutrophil function (Chapter 6), might suggest that activation of Raf may be involved in those functional responses.

Protein phosphatases have previously been shown to regulate apoptosis in a variety of cell types. It has been demonstrated that okadaic acid and calyculin A exert differential effects on apoptosis, for example inducing cell death in leukemic cells (Bøe et al., 1991, Ishida et al., 1992) and inhibiting apoptosis in tumour cell lines (Baxter & Lavin, 1992). Our data illustrate that okadaic acid has a bi-phasic effect upon the rate of eosinophil and neutrophil apoptosis; with lower concentrations inhibiting and higher concentrations promoting granulocyte apoptosis (figure 5.4). In view of reports that okadaic acid inhibits PP2A at lower concentrations and also, PP1 at higher concentrations (Berndt et al., 1987), these data may suggest that the specific inhibition of PP2A regulates inhibition of apoptosis whereas the collective inhibition of both PP2A and PP1, is responsible for inducing the pro-apoptotic effect in these granulocytes. Further experiments investigating the effect of calyculin A (1-100 nM) upon the constitutive rate of neutrophil apoptosis demonstrated that at the concentrations used, this protein phosphatase inhibitor did not modulate apoptosis (figure 5.6). Further experiments are necessary to determine whether lower concentrations of calyculin A will mirror the effect of okadaic acid on neutrophil apoptosis. Furthermore, although these results appear to contradict the bi-phasic effect of okadaic acid on neutrophil apoptosis discussed previously, several differences between these two protein phosphatase inhibitors may account for these discrepancies. Calyculin A has been shown to be a more effective inhibitor than okadaic acid of the catalytic subunit of protein

phosphatase type-1, with reported IC_{50} values for calyculin A of about 2 nM compared with 60-500 nM for okadaic acid (Ishihara et al., 1989). Consequently, calyculin A will collectively inhibit both PP1 and PP2A at a much lower concentration than okadaic acid. It is therefore possible that the concentrations of calyculin A (1-100 nM) used, equate to the concentration of okadaic acid shown to have no effect upon the basal rate of neutrophil apoptosis. Moreover, calyculin A has a similar potency for type 1 and type 2A protein phosphatases (Ishihara et al., 1989), therefore inhibition data with this compound do not discriminate between the enzymes. In support of this hypothesis, the concentration of calyculin A required to inhibit apoptosis (20 nM) in Burkitt's lymphoma cell line BM13674 cells was considerably less than that reported previously for okadaic acid (500 nM) (Song & Lavin, 1993).

We have also presented results illustrating that the protein phosphatase inhibitor, okadaic acid, modulates glucocorticoid-mediated granulocyte apoptosis, with a partial attenuation of the pro-apoptotic effect of dexamethasone in eosinophils (figure 5.5*A*) and a complete attenuation of dexamethasone-mediated inhibition of apoptosis in neutrophils (figure 5.5*B*). In addition, calyculin A (1-100 nM) was also shown to partially attenuate dexamethasone-mediated inhibition of neutrophil apoptosis (figure 5.6*B*). These results obtained using two inhibitors with a different structure and potency, indicate that glucocorticoid-mediated inhibition of neutrophil apoptosis requires the activity of a phosphatase sensitive to both okadaic acid and calyculin A. Collectively these observations are particularly exciting as they suggest that protein dephosphorylation may be an important factor regulating glucocorticoid-induced apoptosis in eosinophils and the down-regulation, by glucocorticoids, of neutrophil

apoptosis. In support of the observed partial attenuation of the pro-apoptotic effect of dexamethasone on eosinophil apoptosis by okadaic acid (0.5 nM), Ohoka et al., (1993) have also demonstrated that okadaic acid inhibits glucocorticoid-induced apoptosis in T cell hybridomas, indicating that protein dephosphorylation is an essential step for glucocorticoid-induced apoptosis in this cell system. While the okadaic acid-sensitive step essential for apoptosis was not biochemically identified, Ohoka and colleagues reported that this step is at the late stage of the apoptotic process since the inhibitory effect of okadaic acid was shown not to be due to either the interference of the translocation of GR or interference of GR binding to GRE or transcription or translation of GRE-regulated genes. Although the mechanism of glucocorticoid-mediated apoptosis is largely unknown, phosphorylation and dephosphorylation has been suggested to influence GR function (Schmidt & Litwack, 1982, Orti et al., 1989). Moreover, GRs are themselves phosphoproteins whose phosphorylation state is quantitatively altered by hormone treatment (Hoeck et al., 1989, Orti et al., 1989) and may therefore be regulated by the protein phosphatases type-1 and type-2A. Furthermore, in the rat GR, there is evidence to suggest that PP2A and/or PP1 may act on a specific subset of phosphorylation sites (DeFranco et al., 1991).

Finally by investigating the effect of calyculin A upon dexamethasone-, LPS-, dibutyryl-cAMP- and GM-CSF-mediated inhibition of neutrophil apoptosis in the same experiment, we have demonstrated that the partial attenuation of dexamethasone-mediated inhibition of neutrophil apoptosis by calyculin A appears to be specific to dexamethasone (figure 5.7). The absence of any modulation by calyculin A of dibutyryl-cAMP-mediated inhibition of neutrophil apoptosis may seem surprising in view

of the abundance of literature regarding the regulation of protein phosphatases by cAMP (Cohen & Cohen, 1989). In the PKA pathway, the balance between PKA activity and opposing protein phosphatases is critical in determining the extent and duration of the response. PKA helps its own cause by phosphorylating I-1, thereby converting it into an inhibitor of PP-1, which is the major protein phosphatase that acts on PKA substrates. In the nucleus, there is a second PP1 inhibitor, NIPP-1, which is in part associated with the catalytic subunit of PP1 (PP1C) and in contrast with PP1, is active in the unphosphorylated state (Beullens et al., 1992). Moreover, unlike I-1, PKA-mediated phosphorylation of NIPP-1 decreases its activity as a PP1 inhibitor and causes dissociation of the PP1C subunit leading to the dephosphorylation of nuclear proteins (figure 5.8). PP2A can reverse this activation and hence specific inhibition of nuclear PP2A with okadaic acid is expected to activate PP1 (Wera & Hemmings, 1995). It has been suggested that the ability of PP2A to reverse PP1C activation may account for why inhibition of PP2A (through okadaic acid treatment) leads to phosphorylation of certain nuclear phosphoproteins (Paulson et al., 1994), although an indirect effect of okadaic acid on protein kinases has not been excluded (Wera & Hemmings, 1995).

One of the major nuclear PKA substrates is the transcription factor CREB (Lalli & Sassone-Corsi, 1994); the phosphorylation of which is discussed in Chapter 6. PP1 and PP2A have been implicated in the dephosphorylation of CREB (figure 5.9), which is accompanied by decreased transcription of cAMP-responsive genes (Hagiwara et al., 1992). In some cells, such as PC-12 cells, PP1 is the major protein phosphatase that dephosphorylates CREB on pSer-133 (Hagiwara et al., 1992), whereas in other cells such as HepG2 hepatoma cells CREB dephosphorylation in nuclear extracts has

been shown to be catalysed by a specific holoenzyme of PP2A (Wadzinski et al., 1993).

It has been suggested that the protein phosphatases responsible for CREB dephosphorylation differ in different cell types, depending on the abundance of PP1 inhibitors and on relative levels of PP1 and PP2A in the nucleus (Hunter, 1995). In addition, PP5, a newly identified member of the PP1/PP2A family, is localised in the nucleus and this protein phosphatase could also play a role in phospho-CREB dephosphorylation (Becker et al., 1994, Chen et al., 1994). The regulation of CREB

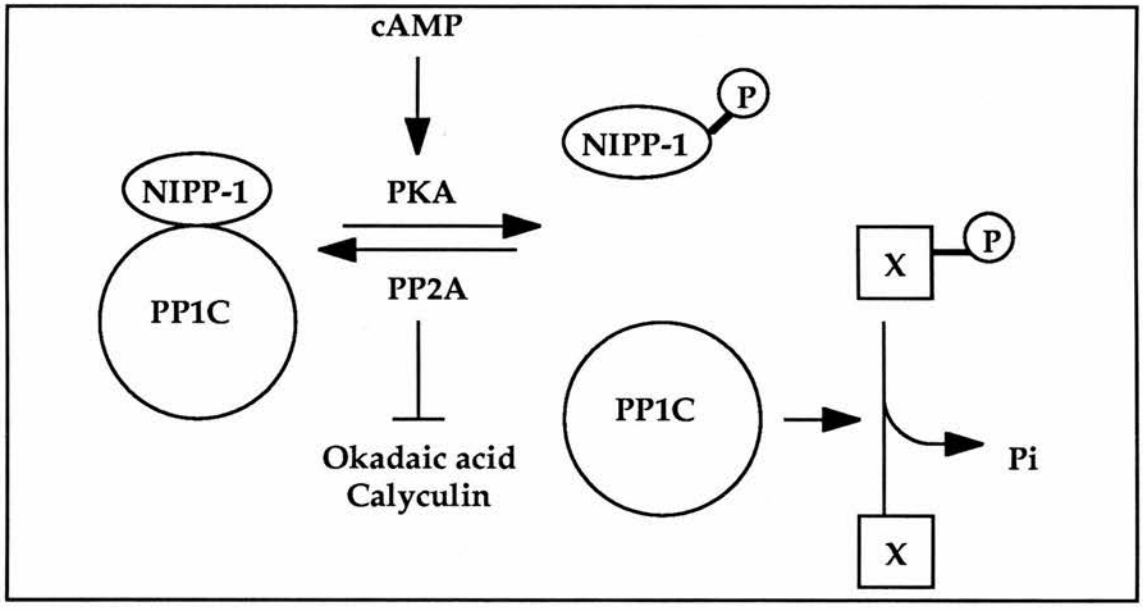

Figure 5.8. A possible mechanism for the control of nuclear PP1
The catalytic subunit (PP1C) of the latent nuclear PP1 enzyme is complexed with the inhibitor NIPP-1. Phosphorylation of NIPP-1 by PKA relieves the inhibition of PP1C, and leads to dephosphorylation of nuclear proteins (X). PP2A reverses the effect. (Diagram modified from Wera & Hemmings, 1995.)

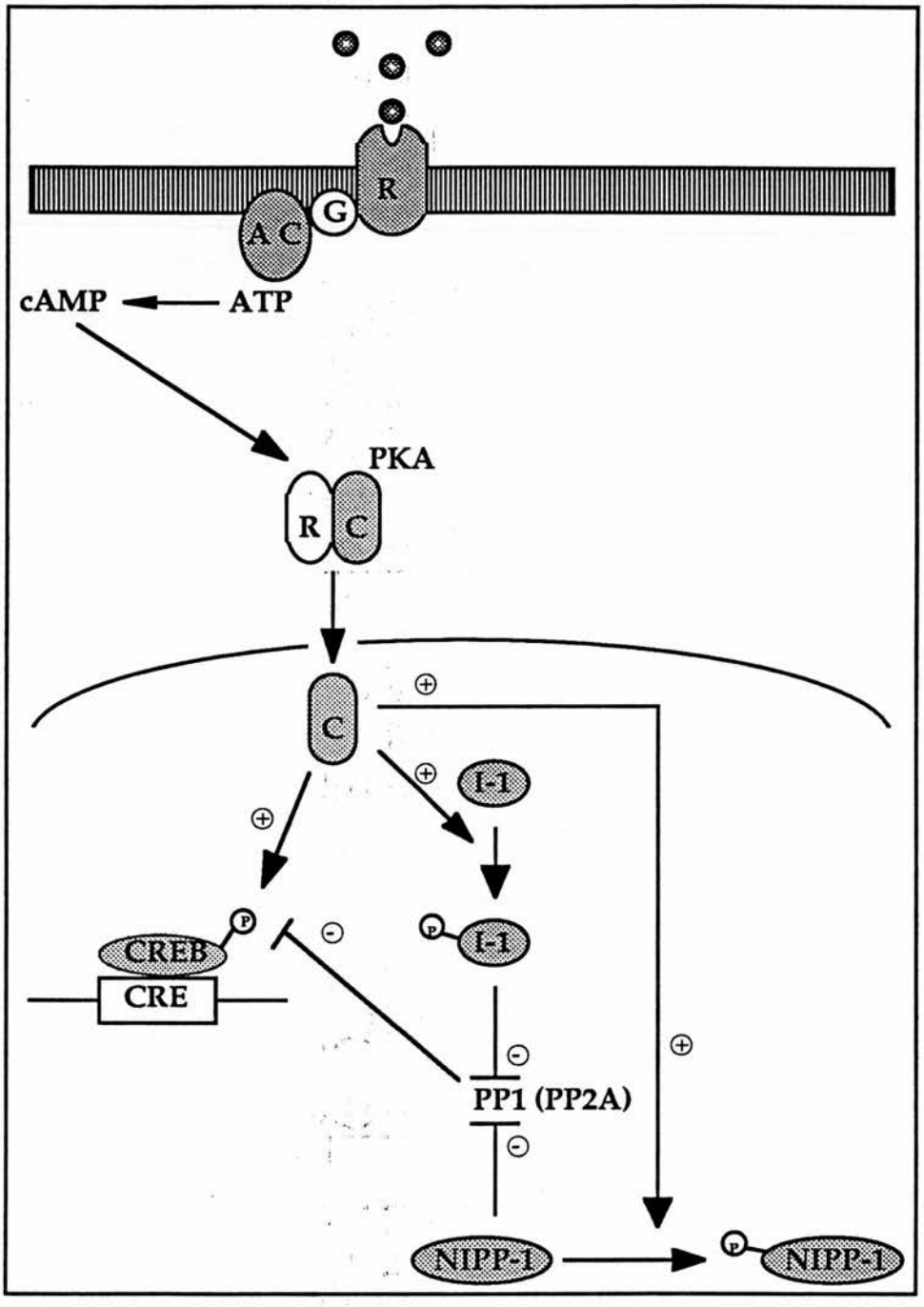

Figure 5.9. A schematic illustration of the protein kinases and protein phosphatases that regulate activation of the CREB transcription factor. Abbreviations: AC, adenylyl cyclase; G, G-proteins; CREB, cyclic AMP -response element binding protein; CRE, cyclic AMP-response element; I-1, protein phosphatase-1 inhibitor; NIPP-1, nuclear protein phosphatase-1 inhibitor. (Modified diagram from Hunter, 1995)

phosphorylation is complicated by the fact that other protein kinases can phosphorylate CREB at Ser-133 and provide cAMP-independent regulation. The Ca^{2+}-calmodulin-stimulated protein kinases, CaMKII and CaMKIV (Dash et al., 1991, Sheng et al., 1991) provide one such example and in principle phosphorylation of these Ca^{2+}-calmodulin-directed kinases could explain how elevated intracellular Ca^{2+} induces CREB Ser-133 phosphorylation and activates CRE-regulated genes such as c-*fos*. Moreover, CaMKII phosphorylates not only the activating Ser-133 but also Ser-142, which inhibits CREB activity (Sun & Tonks, 1994). It has been suggested that this phosphorylation could therefore be responsible for down-regulating CRE-mediated transcription upon elevation of Ca^{2+} in conjuction with PP2B (or calcineurin) which is activated by Ca^{2+}-calmodulin and can dephosphorylate pSer-133 (Hunter, 1995). In contrast to this marked association between cAMP and PP1/PP2A, Gjertsen and colleagues have reported that in IPC-81 cells, a leukemia cell line, okadaic acid and activation of PKA, both of which independently induce apoptosis in this cell line, appear to work through different pathways. They have shown that a mutant IPC subclone, defunct of cAMP response, was as sensitive to okadaic acid as the wild-type IPC cells (Gjertsen et al., 1994). Moreover, the PKA inhibitor H-8 protected against cAMP-induced cell death while enhancing okadaic acid induced death, suggesting synergism. Furthermore, different proteins were induced and phosphorylated in IPC cells exposed to calyculin A, okadaic acid and increased cAMP. These findings are particularly germane when comparing the protein phosphatase inhibitor-mediated attenuation of dexamethasone-induced inhibition of neutrophil apoptosis with the results presented in Chapter 6. It is therefore possible in our system that okadaic acid and calyculin A phosphorylate different substrate proteins to

those phosphorylated by PKA and hence may explain why both protein phosphorylation and dephosphorylation can result in modulation of dexamethasone-mediated inhibition of neutrophil apoptosis.

Finally, the results regarding the involvement of protein phosphatases presented in this chapter should be interpreted with caution. While there is no doubt that increasing evidence suggests a role for phosphatase activity in the regulation of apoptosis, it is possible that the protein phosphatase inhibitors, okadaic acid and calyculin A at higher concentrations (100-1000 nM), in intact cells, may have actions on other protein phosphatases or other as yet unidentified targets. Furthermore, the broad substrate specificity of PP2A and PP1 (Cohen & Cohen, 1989) means that the phosphoprotein(s) presumably mediating the apoptotic process cannot yet be identified using these pharmacological reagents. However some interesting proteins, whose activity state or phosphorylation state are known to be increased by okadaic acid, are the $p34^{cdc2}$/H1 kinase (Yamashita et al., 1990) and the nucleolin fragment, N-60 (Issinger et al., 1988).

In this chapter we have referenced several parallel pathways leading to activation of different subsets of MAPKs, although the extent to which there is cross-talk between these pathways is unknown (Denhardt, 1996). It is however likely that additional signalling cascades remain to be defined in mammalian cells in light of the emerging pathways in yeast, such as MEK5 (English et al., 1995). It is clear, however, that cellular control cannot be achieved by collections of linear signal transduction pathways alone; mechanisms for integrating pathways and generating interactions between pathways are required. The complexity of the

protein phosphorylation and dephosphorylation networks is growing and elucidating the outputs becomes an increasingly daunting task, especially given the cell-type specificities, some of which have been discussed in this chapter. However, the data presented do suggest the involvement of the MAPK/ERK cascade and protein phosphatases in the regulation of some aspects of granulocyte apoptosis.

Chapter 6

The role of PKA (cAMP-dependent protein kinase) in basal and glucocorticoid-mediated granulocyte apoptosis

6.1 Introduction

The ubiquitous role of cAMP as an intracellular 'second messenger' is now well established. This cyclic nucleotide mediates many physiological responses, including growth and differentiation, and has a modulatory effect on many immune mechanisms (Parker, 1979, Kammer, 1988, Haraguchi et al., 1995). Historically, cAMP has been viewed primarily as a negative regulator of immune responses (Kammer, 1988). This has been based on a variety of studies which have demonstrated that cAMP analogs, employed in the high micromolar and low millimolar concentration range, inhibit both B- and T-cell function. More recently, however, cAMP has been shown to enhance a number of immune responses including IL-2 production (Chakkalath & Jung, 1992) and receptor expression (Scholz & Altman, 1989), lymphocyte proliferation (Vazquez et al., 1991, Chakkalath & Jung, 1992, Whisler et al., 1992), antibody production (Gilbert & Hoffmann, 1985) and NF-*k*B activation (Shirakawa & Mizel, 1989). Moreover, several reports suggest that different cell-type subsets, such as in the case of T-cells, possess differential sensitivity to cAMP (Novak & Rothenberg, 1990, Gajewski et al., 1990, Phipps et al., 1991). These observations are indicative of cAMP as both a positive and negative regulatory molecule and it is now generally accepted that in immune responses, cAMP exhibits positive regulatory influences at low concentrations and inhibitory influences at high concentrations (Feuerstein et al., 1996, Koh et al., 1995).

Previous work has established a correlation between an increase in the cAMP level and apoptosis in mammalian epithelial cells and certain plant cells (Pratt & Martin, 1975, Basile et al., 1973). More recent reports have now emerged, also illustrating that elevation of cAMP promotes apoptosis in a variety of cell types including thymocytes (McConkey et al., 1990b, Suzuki et al., 1991, Mastino et al., 1992, Mentz et al., 1995), myeloid cell lines such as IPC-81 (Lanotte et al., 1991), WEH17.2 murine lymphocytes (Dowd & Miesfeld, 1992), ovarian surface epithelial cells (Ackerman & Murdoch, 1993), primary granulosa cells (Aharoni et al., 1995), rat IPC-81 promyelocytic leukemia cells (Vintermyr et al., 1993), and B lymphocytes (Lømo et al., 1995, Brown et al., 1992). However the mechanisms by which cAMP influences granulocyte apoptosis and the effect of cAMP-elevating agents or activation of PKA on these cells, remain ill defined.

Elevation of intracellular cAMP is known to inhibit the activation of many pro-inflammatory immune effector cells (Elwood et al., 1995), including neutrophils, resulting in the suppressed release of eicosanoids and other inflammatory mediators, and the inhibition of neutrophil functions such as agonist-stimulated superoxide release (Kuehl et al., 1987, Schudt et al., 1991a; 1991b) and enzyme release (Rossi & O'Flaherty, 1989). Moreover cAMP can regulate certain eosinophilic functions such as the inhibition of degranulation (Kita et al., 1991b), leukotriene C_4 production (Munoz et al., 1994), thromboxane generation (Souness et al., 1994) and suppress the respiratory burst response of human eosinophils (Dent et al., 1994) and of both guinea pig peritoneal eosinophils (Dent et al., 1991, Souness et al., 1991) and macrophages (Turner et al., 1993). In view of this inhibitory action upon inflammatory cells, it has been

suggested that agents capable of elevating cAMP levels in such cells (e.g., phosphodiesterase (PDE) inhibitors) may have therapeutic potential in inflammatory diseases such as bronchial asthma (Torphy & Undem, 1991, Giembycz, 1992, Giembycz & Dent, 1992). It is now recognised that cyclic nucleotide PDE is not a single enzyme but constitutes a diverse group of structurally distinct proteins, that have been broadly categorised into at least seven isoenzyme families. The involvement of cAMP in the intracellular actions of β adrenoceptor agonists has been well established. PDE inhibition provides an alternative approach to elevating cAMP levels with the aim of combining the bronchodilator actions of β_2-adrenoceptor agonists with anti-inflammatory attributes (Dent & Giembycz, 1996).

We have previously highlighted the importance of apoptosis in the resolution of the inflammatory response (Savill et al., 1989a, Stern et al., 1992), in particular limiting tissue injury and promoting wound repair. In light of the current literature, we investigated the direct effects of cAMP-elevating agents and the role of PKA in basal and glucocorticoid-modulated eosinophil and neutrophil apoptosis.

6.2 Results

6.2.1 Effect of cAMP-elevating agents on the rate of eosinophil and neutrophil apoptosis

Eosinophils ($2x10^6$/ml) cultured in serum-supplemented Iscove's DMEM showed an inhibition in the light microscopic features of apoptosis in the presence of 0.2 mM dibutyryl-cAMP (Figure 6.1*A*). Similarly, treatment of neutrophils ($2x10^6$/ml) with cAMP-elevating agents also inhibited the rate of apoptosis (Figure 6.1*B*). These results indicate that cell permeable cAMP analogs (2 mM dibutyryl-cAMP and 2 mM 8-Br-cAMP) and PG mimetics, specifically the PGD_2 mimetic, ZK 118.182 (30 μM) and the PGE_2 mimetic, 11-deoxy PGE_1 (30 μM), all result in down-regulation of neutrophil apoptosis. Assessment of cell viability and recovery, in both cell types, demonstrated that treatment with these reagents did not significantly alter either of these parameters. Figure 6.2 illustrates the time-course of dibutyryl-cAMP (0.2 mM) and ZK 118.182 (30 μM) induced inhibition of neutrophil apoptosis. After 30 h, the proportion of cells admitting trypan blue in the control population of neutrophils increased rapidly, indicating the onset of secondary cellular necrosis in the 'post-apoptotic' population of cells. However, in the presence of dibutyryl-cAMP and ZK 118.182, cells displayed a high viability (>90%) and the onset of significant necrosis was delayed until the 40 h time period. These results illustrate that agents elevating the intracellular levels of cAMP, inhibit or delay rather than irreversibly block the process of neutrophil apoptosis.

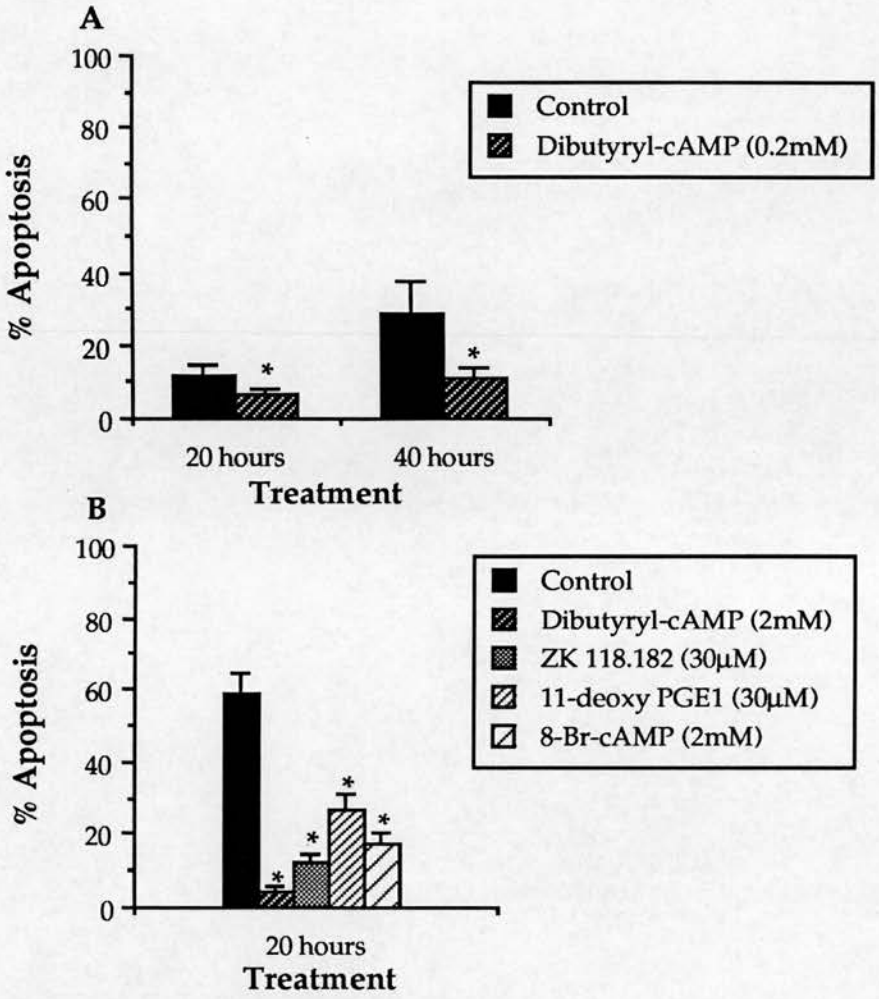

Figure 6.1 Effect of cAMP-elevating agents on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured for 20 and 40 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dibutyryl-cAMP (0.2 mM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or in the presence of the PG mimetics, ZK 118.182 (30 μM) and 11-deoxy PGE_1 (30 μM) or the cAMP analogs, dibutyryl-cAMP (2 mM) and 8-Br-cAMP (2 mM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-6 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

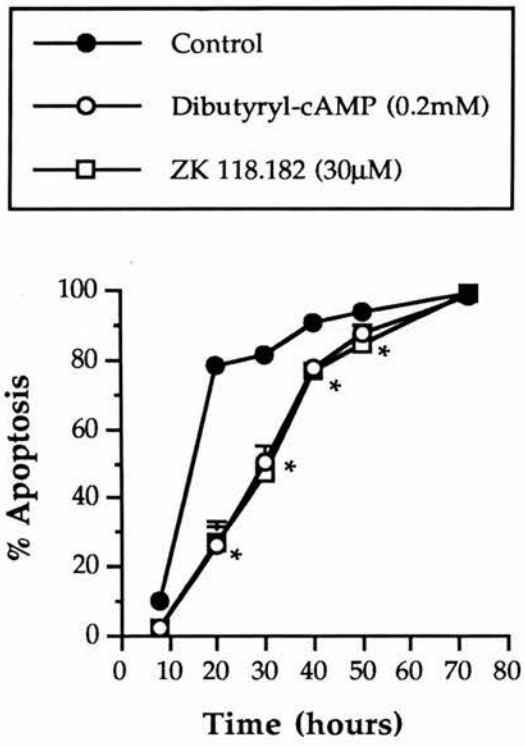

Figure 6.2 Time course for the effect of cAMP-elevating agents on the rate of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or in the presence of dibutyryl-cAMP (0.2 mM) or ZK 118.182 (30 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

6.2.2 Involvement of PKA in eosinophil and neutrophil apoptosis

In light of the findings that cAMP-elevating agents delay apoptosis in both eosinophilic and neutrophilic granulocytes, we were interested in investigating the role of PKA in basal (constitutive) and dibutyryl-cAMP-delayed granulocyte apoptosis. Figure 6.3 illustrates the effect of the PKA inhibitor, H-89, on the rate of eosinophil *(A)* and neutrophil *(B)* apoptosis. H-89 *per se* did not alter the rate of apoptosis in either granulocyte type as compared to the control cell population, suggesting that the basal turnover of cAMP and subsequent activation of PKA may

not be an important factor in regulating the constitutive rate of apoptosis. However, the range of concentrations of H-89 used varied between the two cell types. A high proportion of eosinophils admitted trypan blue when cultured with concentrations of H-89 >10 μM, suggesting that this concentration of the PKA inhibitor was toxic to the cell and therefore induced cell necrosis. In contrast, H-89 (0.01-100 μM) did not induce cellular necrosis in neutrophils with the viability reproducibly being >90% at the time periods indicated in figure 6.4*B*.

The effect of H-89 on dibutyryl-cAMP-mediated inhibition of eosinophil *(A)* and neutrophil *(B)* apoptosis is illustrated in figure 6.4. Initial experiments were carried out, to compare the results obtained from pre-incubating cells with H-89 for 30 min at 37°C before addition of dibutyryl-cAMP, with simultaneous addition or co-culture of these reagents. No significant difference between the results was detected (data not shown). For convenience, all experiments involving H-89 were subsequently carried out by co-culture. H-89 (1-10 μM) was shown to have no effect on dibutyryl-cAMP-induced inhibition of eosinophil apoptosis (figure 6.4*A*), suggesting that cAMP-mediated inhibition of eosinophil apoptosis may not be modulated by PKA activation. In contrast to the effects observed on eosinophils, H-89 (10-100 μM) was shown to attenuate dibutyryl-cAMP-induced inhibition of neutrophil apoptosis in a concentration-dependent manner, with a partial reversal of the inhibitory effect seen at a concentration of 10 μM H-89, followed by a complete reversal of the inhibitory effect at a concentration of 100 μM H-89 (figure 6.4*B*). H-89 (0.01-100 μM) alone was shown to have no effect on the basal rate of neutrophil apoptosis, as previously demonstrated in figure 6.3*B*.

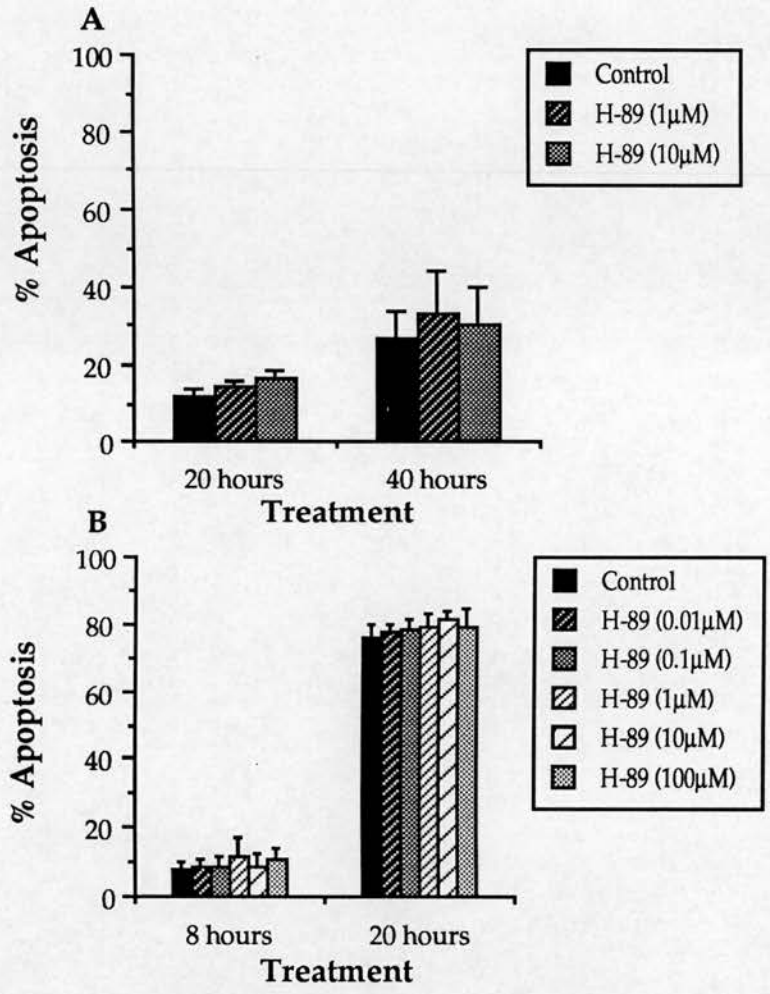

Figure 6.3 Effect of H-89 on the rate of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured for 20 and 40 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of H-89 (1-10 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate. *B*, Neutrophils ($2x10^6$/ml) were cultured for 8 and 20 h, in serum-supplemented Iscove's DMEM either alone (control) or in the presence of H-89 (0.01-100 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate.

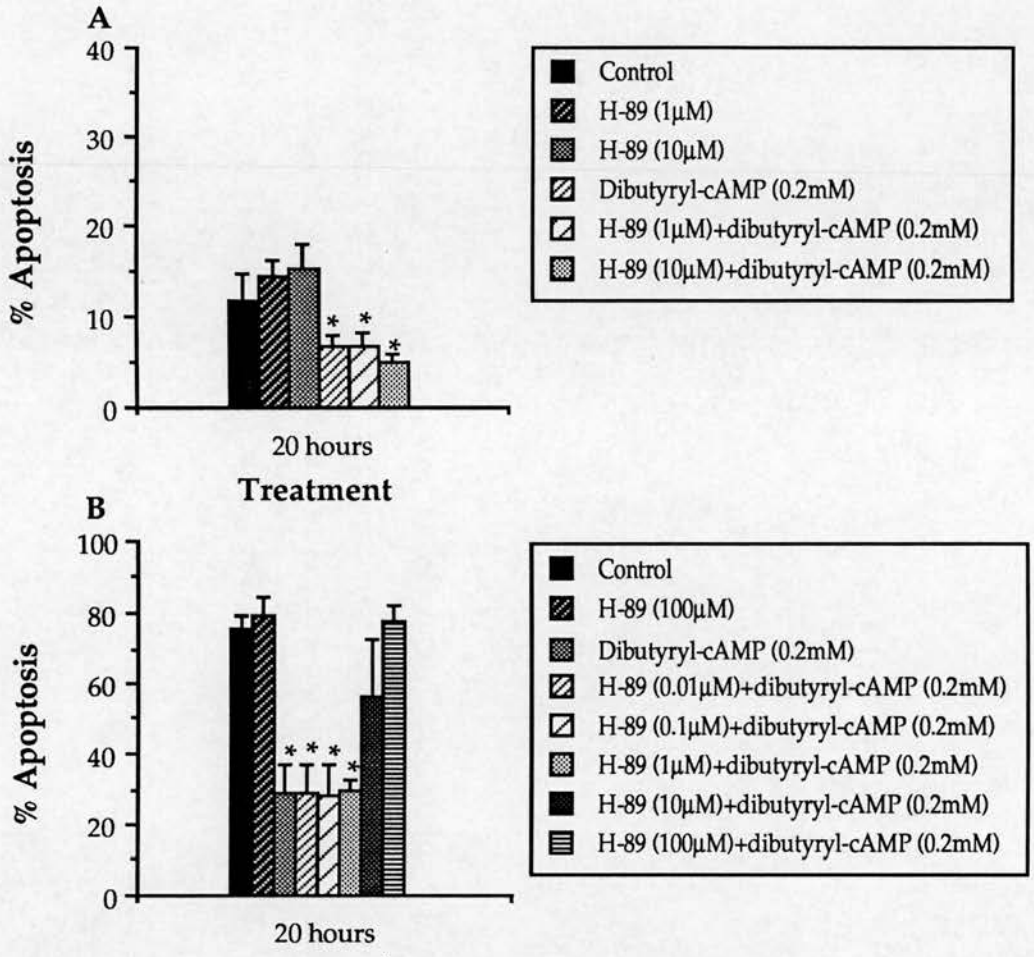

Figure 6.4 Effect of H-89 on dibutyryl-cAMP-mediated inhibition of eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were incubated in serum-supplemented Iscove's DMEM either alone (control) or with dibutyryl-cAMP (0.2 mM), in the presence and absence of H-89 (1-10 μM). Eosinophils were harvested following 20 and 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were incubated for 20 h, in serum-supplemented Iscove's DMEM either alone (control) or with dibutyryl-cAMP (0.2 mM), in the presence and absence of H-89 (0.01-100 μM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

For this reason only one concentration of H-89 (100 μM) alone is illustrated in figure 6.4*B*. These results suggest that cAMP-mediated inhibition of neutrophil apoptosis is signalled by PKA activation. These data are particularly exciting because although cAMP-elevating agents inhibit apoptosis in both eosinophils and neutrophils, the intracellular regulatory mechanisms appear to be different. In eosinophils, our results suggest that elevation of intracellular cAMP levels inhibit apoptosis by a PKA-independent mechanism. However, in contrast cAMP-induced inhibition of neutrophil apoptosis is mediated by a PKA-dependent mechanism.

6.2.3 Involvement of PKA in dexamethasone-mediated eosinophil and neutrophil apoptosis

The intriguing nature of the results illustrated in section 6.2.2 created interest into the possible involvement of PKA as an intracellular regulatory signalling pathway mediating dexamethasone-modulated granulocyte apoptosis. To investigate whether dexamethasone modulates apoptosis via PKA activation, dexamethasone (1 μM) was co-cultured with various concentrations of H-89. Figure 6.5*A* illustrates that H-89 (1-10 μM) does not modulate the pro-apoptotic effect of dexamethasone upon eosinophil apoptosis at the time periods indicated. These results suggest that dexamethasone mediates eosinophil apoptosis via a PKA-independent mechanism. However, in contrast to the effects observed on eosinophils, H-89 (10-100 μM) was shown to attenuate dexamethasone-mediated inhibition of neutrophil apoptosis in a concentration-dependent manner, with a partial reversal of the inhibitory effect seen at a concentration of 10 μM H-89, followed by a complete reversal of the inhibitory effect at a concentration of 100 μM H-89 (figure 6.5*B*). H-89

(0.01-100 μM) alone was shown to have no effect on the basal rate of neutrophil apoptosis, as previously demonstrated in figure 6.3*B*. For this reason only one concentration of H-89 (100 μM) is illustrated in figure 6.5*B*. This pattern of attenuation by H-89 on dexamethasone-mediated inhibition of neutrophil apoptosis mirrors precisely the results illustrated in figure 6.4*B*. Interestingly, at the same concentration that H-89 attenuates dibutyryl-cAMP-induced inhibition of neutrophil apoptosis, this PKA inhibitor also attenuates dexamethasone-mediated inhibition of neutrophil apoptosis. These results suggest that dexamethasone-mediated inhibition of neutrophil apoptosis may be mediated by PKA activation.

6.2.4 Involvement of PKA in LPS-induced inhibition of neutrophil apoptosis

We have demonstrated that both dexamethasone and dibutyryl-cAMP inhibit neutrophil apoptosis and that this inhibitory effect is attenuated by co-culture of these agents with the PKA inhibitior, H-89. To investigate the specificity of these results, LPS (1 μg/ml), another agent known to inhibit the rate of neutrophil apoptosis (Lee et al., 1993) was co-cultured for 20 h in the presence of H-89 (10 μM). The concentration of H-89 (10 μM) that was chosen for use in these experiments was sufficient to observe a partial but significant attenuation of the inhibitory effect mediated by both dexamethasone and dibutyryl-cAMP. Figure 6.6 illustrates that H-89 (10 μM) had no effect on the basal rate of neutrophil apoptosis and did not modulate LPS-induced inhibition of neutrophil apoptosis. However, in the same experiments, H-89 (10 μM) significantly reversed dibutyryl-cAMP- and dexamethasone-mediated inhibition of neutrophil apoptosis.

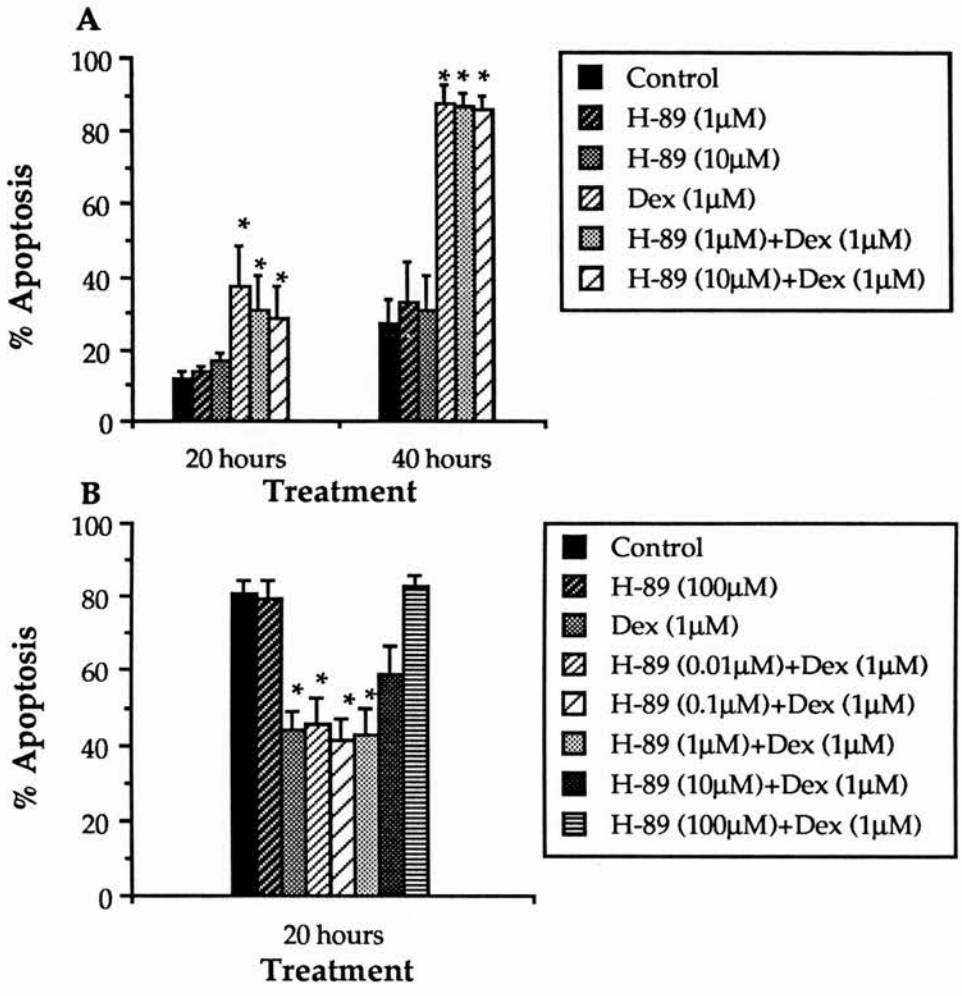

Figure 6.5 Effect of H-89 on dexamethasone-mediated eosinophil (A) and neutrophil (B) apoptosis.

A, Eosinophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of H-89 (1-10 μM). Eosinophils were harvested following 20 and 40 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values). *B*, Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of H-89 (0.01-100 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3-6 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

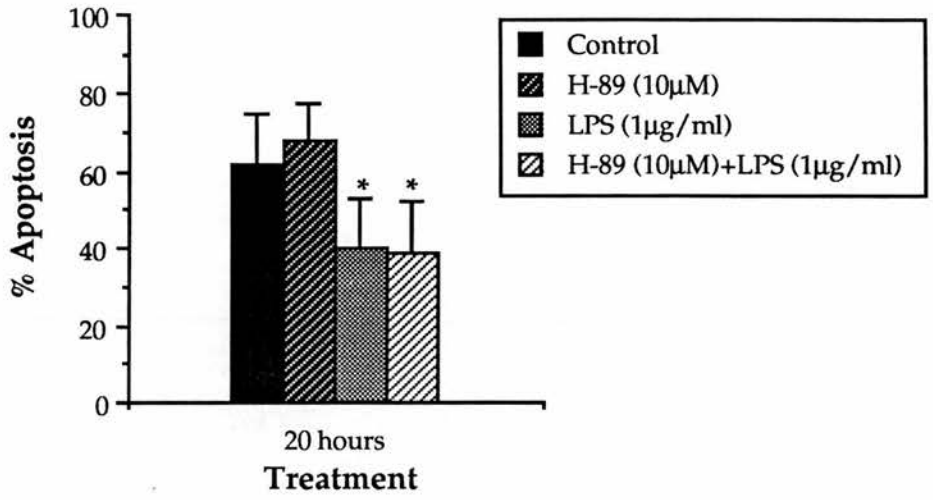

Figure 6.6 Effect of H-89 on LPS-induced inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with LPS (1 μg/ml), in the presence and absence of H-89 (10 μM). Neutrophils were harvested following 20 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

These data argue against the possibility that H-89 has non-specific effects leading to attenuation of all agents that inhibit the rate of neutrophil apoptosis.

6.2.5 Cross-talk between Ca^{2+} and PKA signalling pathways

Given that cAMP-induced thymocyte apoptosis may be a Ca^{2+}-dependent process (McConkey et al., 1990b) and in light of the results presented in Chapter 4, in which the calcium ionophore, A23187 (0.1 μM) was shown to inhibit the rate of neutrophil apoptosis, we investigated whether H-89 would modulate this inhibitory effect, suggesting the possibility of 'cross-talk' between calcium and PKA intracellular signalling pathways. Figure 6.7 illustrates that co-culture of neutrophils with A23187 (0.1 μM) and H-

89 (10 μM) did not modulate the inhibition observed in the presence of A23187 alone. H-89 (10 μM) was again shown to attenuate dexamethasone- and dibutyryl-cAMP-mediated inhibition of neutrophil apoptosis (data not shown).

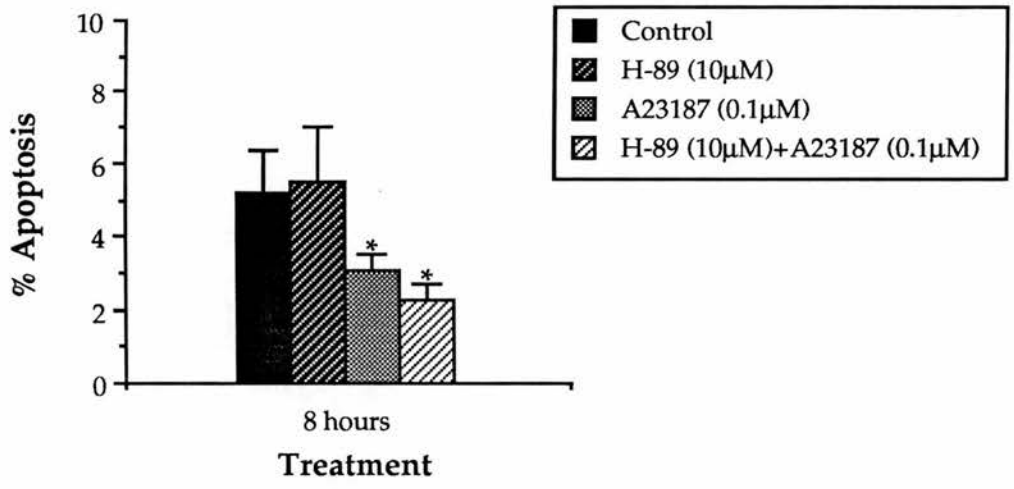

Figure 6.7 Effect of H-89 on A23187-induced inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with A23187 (0.1 μM), in the presence and absence of H-89 (10 μM). Neutrophils were harvested following 8 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

Figure 6.8 illustrates the effect of the intracellular Ca^{2+} chelator, BAPTA/AM (2.5 μM), on dibutyryl-cAMP (0.2 mM)-mediated inhibition of neutrophil apoptosis. The results demonstrate that BAPTA/AM (2.5 μM) did not modulate the inhibition observed in the presence of dibutyryl-cAMP (0.2 mM) alone. An earlier time point of 8 h was chosen for the experiments illustrated in figures 6.7 and 6.8, given that they were carried out as one experiment using the same blood donors, involving use of the reagent BAPTA/AM, which exerts a pro-apoptotic effect upon the rate of neutrophil apoptosis. An earlier time period therefore facilitates easy detection of changes in apoptotic morphology, as explained in Chapter 4 (section 4.2.3). Collectively, these data argue against the possibility of direct 'cross-talk' between calcium and PKA signalling pathways and suggest that these signalling cascades may act independently to regulate neutrophil apoptosis.

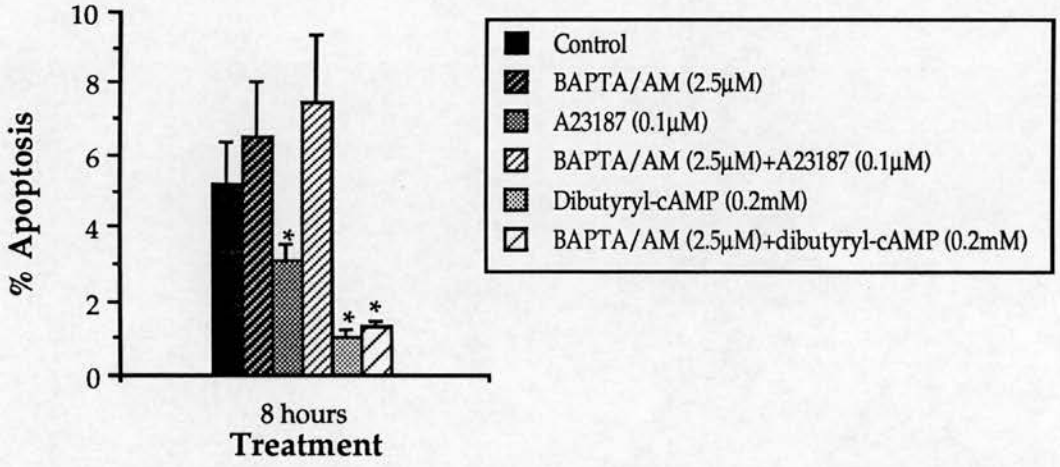

Figure 6.8 Effect of BAPTA/AM on dibutyryl-cAMP-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with A23187 (0.1 μM) and dibutyryl-cAMP (0.2 mM), in the presence and absence of BAPTA/AM (2.5 μM). Neutrophils were harvested following 8 h in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

6.2.6 Effect of dibutyryl-cAMP on dexamethasone-mediated inhibition of neutrophil apoptosis

We have reported the dramatic inhibitory effects that both dexamethasone and dibutyryl-cAMP individually induce on the rate of neutrophil apoptosis. To investigate whether a common inhibitory mechanism is used by these agents, we studied the effect of co-culturing dibutyryl-cAMP and dexamethasone on the rate of neutrophil apoptosis. Figure 6.9 shows that when neutrophils are cultured in the presence of both dexamethasone and dibutyryl-cAMP their individual inhibitory effects are less than additive, suggesting that these reagents inhibit neutrophil apoptosis possibly by the same pathway. However, further experiments involving much wider concentration ranges of both agents are necessary to make any firm conclusions regarding these data.

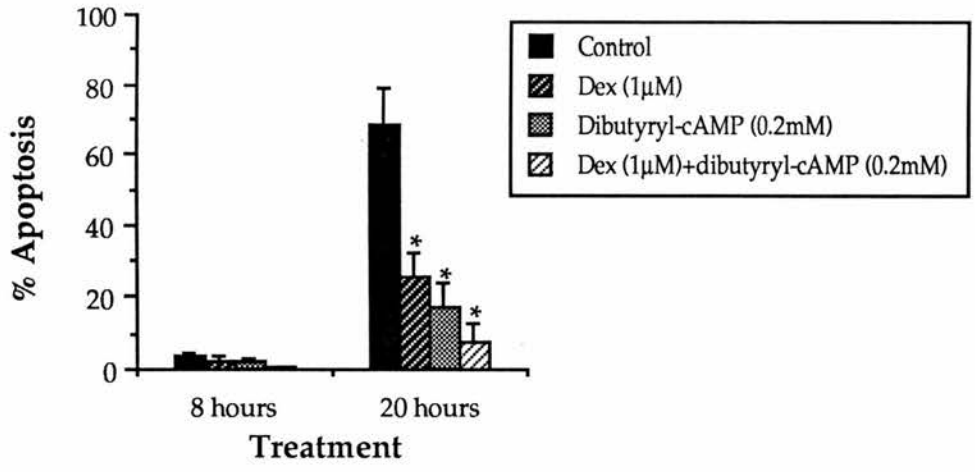

Figure 6.9 Effect of dibutyryl-cAMP on dexamethasone-mediated inhibition of neutrophil apoptosis.
Neutrophils ($2x10^6$/ml) were cultured for 8 and 20 h, in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM), in the presence and absence of dibutyryl-cAMP (0.2 mM). At the time periods indicated, cells were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

6.2.7 Measurement of PKA activity in neutrophils

Using the pharmacological PKA inhibitor, H-89, we have presented results suggesting that dexamethasone-mediates the inhibition of neutrophil apoptosis by a PKA-dependent mechanism. We therefore investigated the A-kinase isoenzyme profile present within neutrophils and measured PKA activity directly in response to dexamethasone treatment. A-kinase isoenzymes present in the soluble fraction of freshly isolated neutrophils were separated by anion-exchange chromatography over Q-sepharose and the relative proportions of each class were determined using kemptide as a substrate (Giembycz & Diamond, 1990). The specific eicosa-peptide A-kinase inhibitor (IP_{20}) was used to ensure that kemptide was phosphorylated specifically by soluble PKA (Cheng et al., 1986), as indicated by the ability of IP_{20} to abolish cAMP-stimulated phosphokemptide formation (figure 6.10). Thus, figure 6.10 illustrates that analysis of cAMP-stimulated, IP_{20}-sensitive phosphotransferase activity revealed two main peaks eluting at 128 mM (type I) and 216 mM NaCl (type II), with the approximate type I:type II isoenzyme percentage ratio being 73:27 respectively. The observation that human neutrophils contain a mixture of both type I and type II A-kinase isoenzymes, suggested that in the PKA assay method, buffers with an intermediary ionic strength of 100 mM would be optimal for the dissociation and reassociation kinetics of A-kinase (see *Discussion*). The subcellular distribution of cAMP-dependent protein kinase in neutrophils is shown in table 6.1 and these results illustrate that the intracellular distribution of cAMP-dependent protein kinase is most enriched in the cytosolic (soluble) fraction.

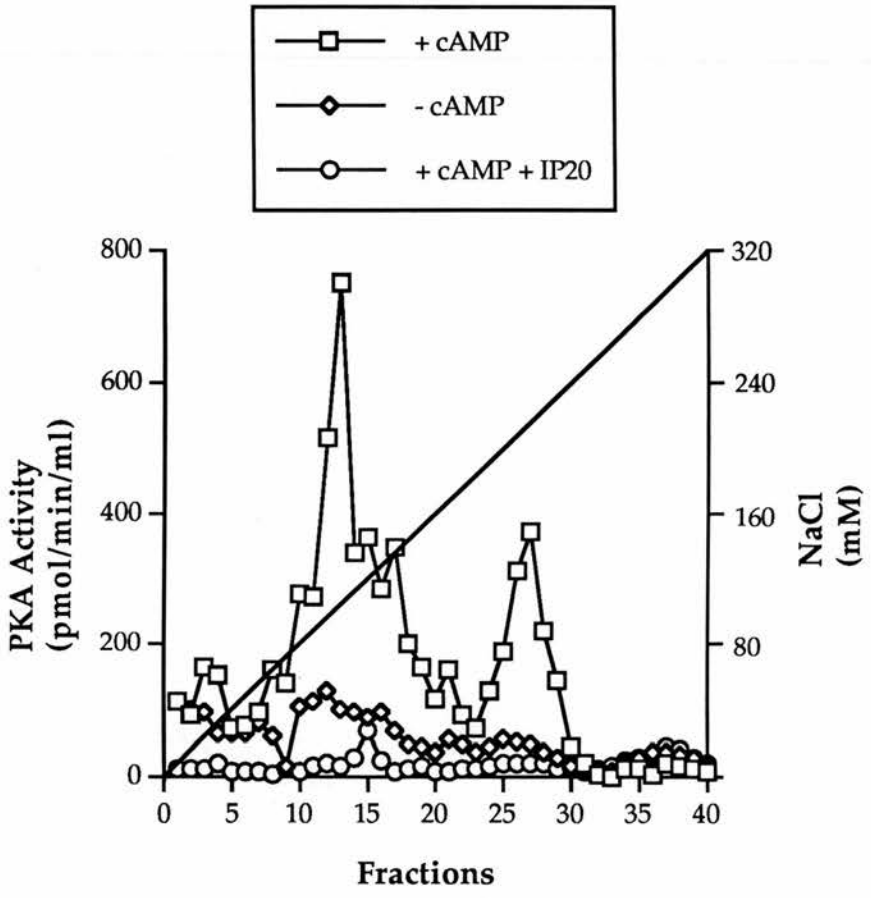

Figure 6.10 A-kinase isoenzyme elution profile of the cytosol fraction of neutrophils.

Lysates of freshly isolated neutrophils ($200x10^6$) were applied to a column of Q-sepharose and A-kinase enzymes were eluted with a linear NaCl gradient, running from 0 to 320 mM. IP_{20}-sensitive (o) A-kinase activity was then estimated in the absence (◊) and presence (□) of cyclic AMP (10 μM) using kemptide as the substrate, as described in Materials and Methods (Chapter 2). Values denoted by the symbols refer to pmol of [γ-^{32}P]ATP transferred to kemptide per minute per ml. The results presented are from a representative experiment.

Fraction	[γ-^{32}P]ATP Transferred (pmol/min/10^6 cells)
Soluble	76.3 ± 28.0
Particulate	48.6 ± 26.0

Table 6.1 Subcellular distribution of cAMP-dependent protein kinase in neutrophils.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM alone. After 20 h, cells were harvested and divided into 2 samples. Sample (1) was lysed for 30 min in buffer A containing 1% Triton X-100, to form the soluble fraction and sample (2) was lysed for 30 min in buffer A in the absence of Triton X-100. Sample (2) was then centrifuged (31000 g, 30 min) at 4°C, to form the particulate fraction. A-kinase activity was then estimated for the soluble and particulate untreated fractions, as described in Materials and Methods (Chapter 2). Data represent mean ± SEM of 3 separate experiments, each performed in triplicate.

Figure 6.11 illustrates the effect of dexamethasone on the activation state of PKA. Treatment of neutrophils with dexamethasone (1 μM for 20 h) increased the basal PKA activity ratio from 0.33 ± 0.05 to 0.41 ± 0.06, ($p<0.05$, n=4). Similarly, neutrophils co-cultured in the presence of PGE_2 (1 μM) and IBMX (100 μM), as a positive control, for 1 h, after 19 h in culture previously untreated (from 19 to 20 h), increased the basal PKA activity ratio from 0.33 ± 0.05 to 0.69 ± 0.03, ($p<0.05$, n=4). The percentages of apoptosis in identical cultures were assessed morphologically as: control, 52.8 ± 7.1%; dexamethasone 22.0 ± 5.3%.

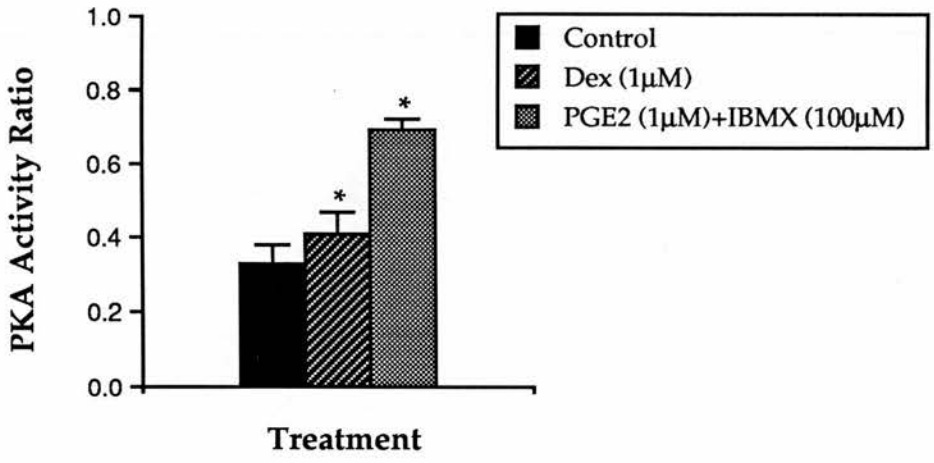

Figure 6.11 Effect of dexamethasone on the PKA activity ratio of neutrophils.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone (control) or with dexamethasone (1 μM) for 20 h, or alternatively in the presence of both PGE_2 (1 μM) and IBMX (100 μM) for 1h (from 19 to 20 h). Neutrophils were harvested following 20 h in culture and the PKA activity ratio was subsequently estimated as described in Materials and Methods. Data represent mean ± SEM of 4 separate experiments, each performed in triplicate (* $p<0.05$ compared with control values).

These results illustrate that dexamethasone may inhibit neutrophil apoptosis by a mechanism involving an increase in PKA activity. Moreover the results expressed in table 6.2 show that the increase in the PKA activity ratio is specifically mediated via the glucocorticoid receptor, since RU38486 (10 μM) attenuated the increase in PKA activity induced by dexamethasone.

Treatment	PKA Activity Ratio (pmol/min/10^6 cells)
Control	0.28 ± 0.01
Dexamethasone (1 μM)	0.33 ± 0.04
PGE_2 (1 μM) + IBMX (100 μM)	0.73 ± 0.37
RU38486 (10 μM)	0.29 ± 0.01
RU38486 (10 μM) + Dexamethasone (1 μM)	0.25 ± 0.04

Table 6.2 Effect of co-culturing neutrophils with RU38486 and dexamethasone on the PKA activity ratio.
Neutrophils ($2x10^6$/ml) were cultured in serum-supplemented Iscove's DMEM either alone or in the presence of dexamethasone (1 μM for 20 h), PGE_2 (1 μM) and IBMX (100 μM) (for 1 h, after 19 h in culture previously untreated), RU38486 (10 μM for 20 h) or co-cultured with RU38486 and dexamethasone (10 μM and 1 μM respectively for 20 h). After 20 h, cells were harvested and the PKA activity ratio was subsequently estimated as described in Materials and Methods (Chapter 2). Data represent mean of an individual experiment, performed in triplicate.

6.3 Discussion

We have demonstrated that cAMP-elevating agents inhibit granulocyte apoptosis (figure 6.1), whereas in a number of other cell types increases in the intracellular levels of cAMP have been associated with the induction of apoptosis (McConkey et al., 1990b, Lanotte et al., 1991). In the case of the neutrophil, we have illustrated that elevation of cAMP, through independent mechanisms, using cell permeable cAMP analogs (dibutyryl-cAMP and 8-Br-cAMP), receptor agonists (ZK 118.182 and 11-deoxy PGE_1) and direct activation of adenylyl cyclase (forskolin), all result in the down-regulation of neutrophil apoptosis (*Results* and Rossi et al., 1995).

Dibutyryl-cAMP-induced inhibition of eosinophil apoptosis was found to be mediated by a mechanism that was insensitive to H-89 and therefore PKA-independent (figure 6.4*A*). H-89 alone had no significant effect upon the rate of eosinophil apoptosis at concentrations up to 10 μM, suggesting that basal turnover of cAMP may not be an important factor in regulating eosinophil apoptosis. However, since we have not identified a positive control showing that H-89 is accessing and functioning in eosinophils, these data should be interpreted with caution. Nevertheless, these observations are consistent with the recent findings by Hallsworth and colleagues, who have reported that cholera toxin, which elevates cAMP by interacting with Gs and dibutyryl-cAMP, a cell permeable analog of cAMP, prolonged eosinophil survival in the absence of GM-CSF by a PKA-independent mechanism (Hallsworth et al., 1996). Moreover, they demonstrated that these cAMP-elevating agents have the ability to overcome the protective effect of GM-CSF on eosinophil apoptosis, suggesting that cAMP is able to interfere with the anti-apoptotic signals induced by GM-CSF. The mechanism of inhibition of GM-CSF-induced

cell survival by cAMP, was shown to be PKA-dependent since addition of H-89 to eosinophils cultured with GM-CSF and cholera toxin attenuated the effect of cholera toxin on eosinophil survival. However, the mechanism by which GM-CSF prolongs eosinophil survival was shown not to be related to detectable reductions in cAMP levels within the cell.

Although PKA is known to mediate many of the biological responses of cells to cAMP, PKA-independent events have been reported involving the direct binding of cAMP (Piper et al., 1993, DiFrancesco & Tortora, 1991) and there is also evidence that cAMP can activate PKG (Lincoln et al., 1990). However, Hallsworth and colleagues have shown in their study, that dibutyryl-cGMP, a cell permeable cGMP analog, did not affect eosinophil survival under any condition, indicating that cAMP does not mediate its effects by activation of PKG in human eosinophils (Hallsworth et al., 1996). Similarly in neutrophils, dibutyryl-cGMP (0.2 mM) does not affect the control rate of apoptosis or indeed modulate dexamethasone-mediated neutrophil apoptosis (n=3): control, 52.7 ± 17.5%; dibutyryl-cGMP (0.2 mM), 52.3 ± 18.3%; dexamethasone (1 μM), 22.6 ± 11.1%; co-culture of dibutyryl-cGMP (0.2 mM) and dexamethasone (1 μM), 21.1 ± 7.0%. The mechanism underlying cAMP-mediated inhibition of eosinophil apoptosis is therefore unknown and is an exciting finding requiring further work.

In contrast, dibutyryl-cAMP-induced inhibition of neutrophil apoptosis was found to mediated by a mechanism that was sensitive to H-89 and therefore probably PKA-dependent (figure 6.4*B*). H-89 alone had no significant effect upon the rate of neutrophil apoptosis suggesting that the basal turnover of cAMP may not be important in the regulation of

neutrophil apoptosis. In summary, we have shown that cAMP-elevating agents inhibit eosinophil apoptosis by a PKA-independent mechanism and inhibit neutrophil apoptosis by a PKA-dependent mechanism. These results illustrate yet again that eosinophils and neutrophils are regulated differently.

We were therefore interested in investigating the effect of H-89 on glucocorticoid-mediated granulocyte apoptosis (figure 6.5). Interestingly, the same pattern of PKA-independency in eosinophils and dependency in neutrophils was observed. In eosinophils, the pro-apoptotic effect of dexamethasone was shown to be insensitive to H-89, therefore suggesting that dexamethasone up-regulates eosinophil apoptosis by a PKA-independent mechanism. However, this mechanism could still involve dexamethasone-induced alterations in cAMP levels within the cell, since dibutyryl-cAMP-modulated eosinophil apoptosis was also shown to be PKA-independent. In neutrophils, the anti-apoptotic effect of dexamethasone was shown to be sensitive to H-89 and was attenuated at concentrations of H-89 $\geq$ 10 μM. This result is particularly exciting and suggests that the mechanism of dexamethasone-mediated inhibition of neutrophil apoptosis may be PKA-dependent. The PKA-dependency of glucocorticoid-mediated inhibition of neutrophil apoptosis was shown to be specific to dexamethasone since other agents known to inhibit neutrophil apoptosis such as LPS (figure 6.6) and the calcium ionophore, A23187 (figure 6.7) were not sensitive to H-89. Moreover, we have illustrated that in the neutrophil there is no evidence of direct cross-talk between Ca^{2+} and cAMP-dependent intracellular signalling pathways governing apoptosis (figures 6.7 and 6.8). This is in contrast to cAMP-induced thymocyte apoptosis, which although does not involve an

increase in intracellular Ca^{2+} levels, is a Ca^{2+}-dependent process, as the presence of intracellular Ca^{2+} chelators inhibits the cAMP effect (McConkey et al., 1990b). Moreover, reported synergistic chloride secretory responses as a result of combined treatment with agents that increase either cAMP or cytosolic Ca^{2+}, in T_{84} human colonic epithelial cells and rat pancreatic acinar cells, suggest cross-talk between Ca^{2+} and cAMP-dependent signalling pathways at the level of second messenger generation (Vajanaphanich et al., 1995). Furthermore, less than additive inhibition of neutrophil apoptosis observed in the presence of both dibutyryl-cAMP and dexamethasone (figure 6.9), suggests that these reagents inhibit neutrophil apoptosis by the same mechanism. Although we have not directly monitored cAMP levels, these data may suggest that dexamethasone-mediates neutrophil apoptosis by PKA activation. In support of this hypothesis, cAMP elevation by PGE_2 or forskolin, and the glucocorticoid, methylprednisolone have been reported to enhance each others effects on DNA fragmentation and viability in mouse thymocytes (McConkey et al., 1993).

The formation of cAMP within a cell results from the complex interaction of receptors, G-proteins and adenylyl cyclase (Levitzki, 1988). Interactions among the components of this signalling cascade dictate the magnitude of the intracellular formation of cAMP, and thereby provide several loci where glucocorticoids may exert their regulatory influence. For example, the gene for the human β_2-adrenoceptor which is expressed in all types of lymphocytes to stimulate cAMP formation (Brodde et al., 1987, Maisel et al., 1989) contains a glucocorticoid response element (Hadcock et al., 1989) and is therefore a potential loci where glucocorticoids may exert their regulatory influence. Glucocorticoids can

enhance expression of the α-subunit of the adenylyl cyclase-stimulating G-protein, Gs, in various non-lymphoid cell lines including ROS 17/2.8 osteosarcoma cells (Rodan & Rodan, 1986), opossum kidney (OK) cells (Rizzoli & Bonjour, 1987) and rat pituitary (GH_3) cells (Chang & Bourne, 1987). Based on these observations, it has been hypothesised that cAMP may play a role in the immunosuppressant effects of glucocorticoids (Gruol et al., 1989, Michel & Brodde, 1989). Interestingly, Michel and colleagues demonstrated that dexamethasone sensitizes cAMP formation in resting lymphocytes by altering adenylyl cyclase, resulting in an enhanced capability of cAMP generating agonists to inhibit early steps of lymphocyte activation (Michel et al., 1994). Dexamethasone reportedly enhanced the maximal adenylyl cyclase responses to a similar extent for a range of agonists tested. However since forskolin (a direct adenylyl cyclase catalyst activator which also has some G-protein effects) and $MnCl_2$ (a direct adenylyl cyclase activator) stimulate adenylyl cyclase distal to receptors (as assessed using the receptor agonist, PGE_2) and G-proteins (as assessed using GTP as a direct G-protein activator), these authors concluded that dexamethasone treatment might primarily affect adenylyl cyclase. This study also directly investigated the effect of dexamethasone treatment on lymphocyte β_2-adrenoceptor and G-protein α-subunit expression, producing results that did not significantly alter the expression of β_2-adrenoceptors or G-protein α-subunits, namely G_s and G_i. Michel and colleagues also reported that PGE_1-, GTP- and forskolin-stimulated adenylyl cyclase activity is also enhanced in lymphocytes obtained from patients treated with glucocorticoids thus providing evidence that the mechanisms discussed above may also operate in a clinical situation. These findings are of particular interest in light of our

results and may provide possible clues to the loci where dexamethasone exerts its regulatory effect upon neutrophil apoptosis.

Further evidence substantiating our hypothesis that dexamethasone mediates inhibition of neutrophil apoptosis by a PKA-dependent mechanism was obtained by A-kinase isoenzyme elution (figure 6.10) and from the direct measurement of PKA activity in dexamethasone treated neutrophils, aged for 20 h in culture (figure 6.11). Performing the A-kinase isoenzyme elution profile was particularly important to determine which PKA isoenzymes are present in human neutrophils; a parameter that has not been fully determined to our knowledge, thereby allowing efficient measurement of PKA activity. Human neutrophils were shown to contain a mixture of both type I and type II A-kinase isoenzymes as assessed by Q-sepharose column chromatography, where A-kinase isoenzymes were eluted with a linear NaCl gradient. Cyclic AMP activates PKA through the formation of a regulatory subunit (R).cAMP.catalytic subunit (C) complex (Palmer et al., 1980) and the subsequent dissociation of this ternary complex produces active catalytic subunits.

Equation (1)

$$R_2C_2 + 4cAMP = R_2.4cAMP.C_2 = R_2.4cAMP + 2C$$

Direct measurement of PKA activity is expressed as an 'activity ratio' and is derived from assessment of the cAMP-dependency of the protein kinase (i.e., activity minus cAMP/activity plus cAMP) (Corbin et al., 1973). Validity of the protein kinase activity ratio depends upon total inhibition of both the forward (i.e., dissociation) and backward (i.e., reassociation) reactions, illustrated in Equation 1, being maintained during the PKA

assay. Various procedures have therefore been employed to ensure that this inhibition is maintained (Palmer et al., 1980).

(1) Reassociation of type II protein kinase isoenzyme is blocked by use of a high NaCl concentration

(2) Reassociation of type I isoenzyme is blocked by addition of the phosphodiesterase inhibitior IBMX and a low NaCl concentration is used and

(3) Cells shown to contain both type I and type II isoenzymes require the use of an intermediary NaCl concentration in addition to the presence of IBMX.

The A-kinase isoenzyme elution profile (figure 6.10) demonstrating the presence of both type I and type II isoenzymes in neutrophils, was therefore suggestive of an intermediary NaCl concentration used in the presence of IBMX; providing optimum conditions for the direct measurement of PKA activity, by inhibiting the breakdown of cAMP and thereby maintaining the position of the equilibrium of the reaction shown in Equation (1). The subcellular distribution of cAMP-dependent protein kinase in neutrophils (table 6.1) was shown to be most enriched in the cytosol. These data indicate that the cellular distribution of PKA is not random. Moreover, a major group of anchoring proteins (AKAPs) that target the RII-containing protein kinase to specific subcellular locations has now been identified (Carr et al., 1991, Coghlan et al., 1993, Scott & McCartney, 1994).

Direct measurement of the effect of glucocorticoids on the activation state of PKA in neutrophils demonstrated a significant increase in the PKA activity ratio as compared to that of control cells (figure 6.11). Moreover,

dexamethasone was shown to both increase the PKA activity ratio and inhibit apoptosis, as assessed morphologically, in identical cultures of neutrophils. These data are extremely interesting and support the pharmacological data discussed previously. However, the observed increase in the PKA activity ratio may simply reflect the lower rate of apoptosis in dexamethasone-treated neutrophils as compared to the control population of cells. Further experiments performed at earlier time periods (e.g., 8 h), when the dexamethasone-induced alteration in the rate of neutrophil apoptosis is far less marked, may help to validate these findings.

Neutrophils were cultured in the presence of dexamethasone for the usual 20 h time period, before direct measurements of PKA activity were carried out (figure 6.11). However, cells co-cultured with PGE_2 and IBMX, as a positive control for the experiment, were incubated for 20 h in total, with the addition of PGE_2 and IBMX for only 1 h, from the time period 19 h to 20 h. This precaution was taken in light of recent reports demonstrating that prolonged elevation of intracellular cAMP leads to up-regulation of phosphodiesterases, thereby resulting in an increased degradation of cAMP and an actual decline in intracellular cAMP levels. This has been shown to be the case in human monocytic cell lines, with prolonged elevation of cAMP leading to the up-regulation of PDE IV (Dent & Giembycz, 1996) and also in LRM55 glial cells which have been shown to produce a rapid increase followed by a slow decline in intracellular cAMP levels in response to continuous stimulation with β-adrenoceptor agonists (Madelian et al., 1985). The presence of PDE inhibitors during treatment abolished this decline in cAMP contents, suggesting that the apparent 'desensitization' observed in these cells

might reflect an increase in the degradation of cAMP rather than a decline in its synthesis (Madelian & Shain, 1987).

Recently, there has been an intense interest in the potential usefulness of PDE inhibitors for the treatment of inflammatory diseases (Torphy & Undem, 1991, Giembycz, 1992, Nicholson & Shahid, 1994). The distribution of PDE isoenzymes in inflammatory cells has now been extensively studied and the prominant PDEs expressed are the type III and IV isoenzymes (Nicholson & Shahid, 1994). Interestingly, eosinophils and neutrophils have been reported to contain only PDE IV (Dent & Giembycz, 1996). *In vitro* studies have established that inhibition of these isoenzymes by selective PDE inhibitors, suppresses a variety of inflammatory cell functions including oxygen radical generation and secretion of granule proteins and cytokines (Dent & Giembycz, 1996). Moreover, Elwood and colleagues have demonstrated that PDE isoenzyme inhibitors possess inhibitory activity *in vivo* (Elwood et al., 1995). Rolipram (a type IV PDE isoenzyme inhibitor) and Org 20241 (a combined type III and IV inhibitor) were shown to inhibit allergen-induced eosinophil and neutrophil accumulation into the lungs of ovalbumin-sensitized Brown-Norway rats. This *in vivo* model was chosen as it mimics several of the features of asthma in man. Interestingly, these data support a potential anti-inflammatory role of PDE IV inhibitors in this species. Furthermore, glucocorticoids have also been shown to inhibit eosinophil accumulation in this model (Elwood et al., 1992). In addition, rolipram has been shown to inhibit neutrophil apoptosis (Rossi et al., personal communication) which may offer another possible mechanism by which glucocorticoids regulate apoptosis and co-ordinate their anti-inflammatory actions.

Although the mechanisms by which cAMP influences neutrophil apoptosis and PKA (or cAMP-dependent protein kinase) influences glucocorticoid-mediated neutrophil apoptosis are unknown, the expression of key regulatory genes may be involved. Downstream effects of cAMP on gene expression are thought to be mediated through PKA phosphorylation of the transcription factor, CREB, at Ser-133 (Gonzalez & Montminy, 1989) (figure 6.12). CREB is a member of a diverse family of DNA-binding proteins, whose phosphorylation results in the transcriptional activation of various genes that contain cAMP response elements (CREs) in their promoter or enhancer regions (Hoeffler et al., 1988, Gonzalez & Montminy, 1989, Sheng et al., 1991). Glucocorticoid receptors offer yet another mechanism by which an external signal may be transduced into the nucleus. The binding of these hormones to their receptors causes a conformational change of the proteins which allows them to recognise hormone response elements (HREs) in the DNA sequence of inducible genes (Yamamoto, 1985, Beato, 1989). The mechanisms underlying dexamethasone-mediated inhibition of neutrophil apoptosis may therefore also involve a synergistic interaction between the glucocorticoid receptor (GR) and CREB hormone-responsive transcription factors, acting in concert to regulate gene transcription. Studies investigating the effects of PKA inhibitors on glucocorticoid receptor numbers and glucocorticoid receptor translocation and binding to GREs by electrophoretic mobility shift assays (EMSA), may elucidate further the specific mechanisms governing dexamethasone-mediated inhibition of neutrophil apoptosis.

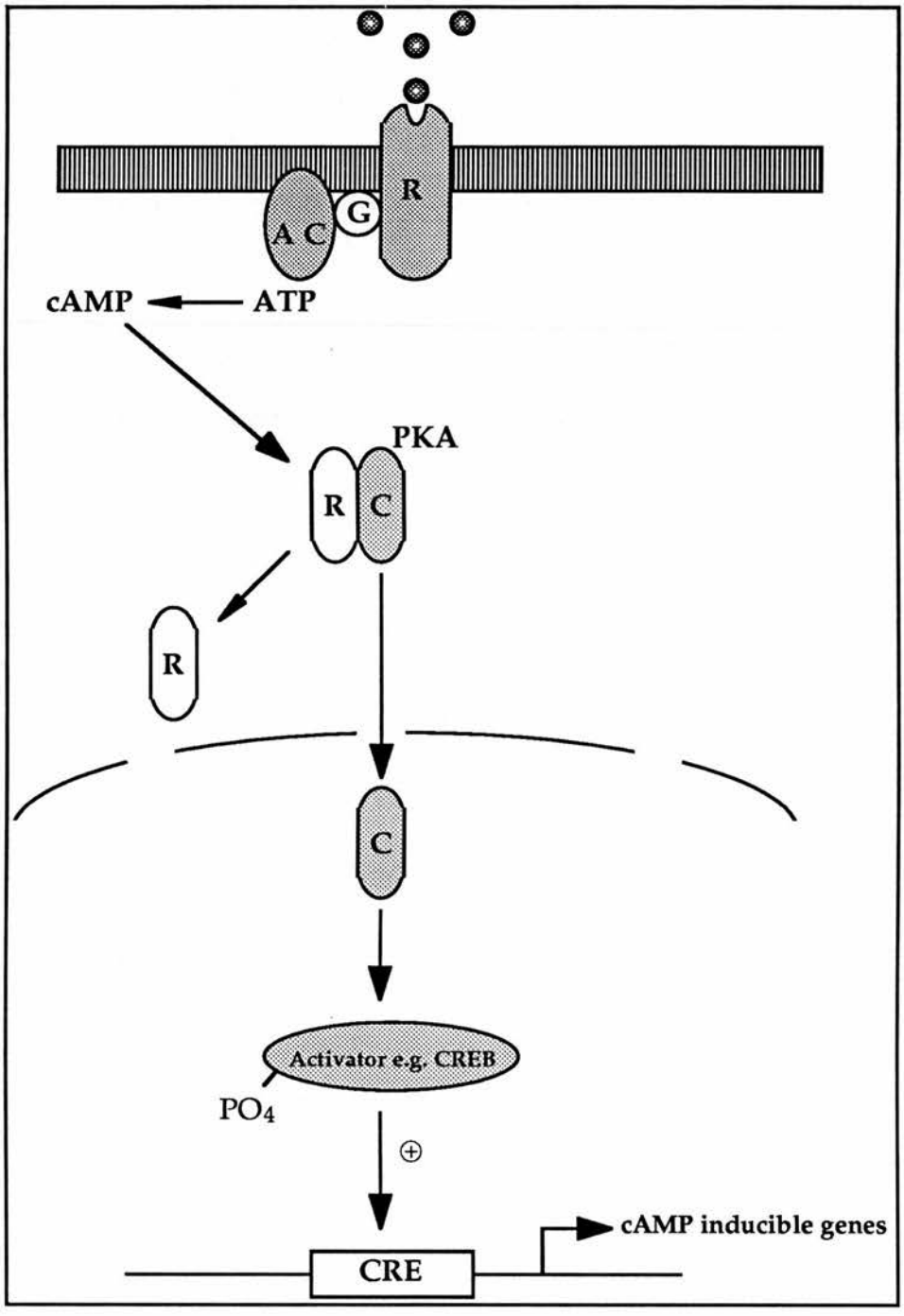

Figure 6.12 Schematic diagram illustrating the cAMP/PKA signal transduction pathway.
Ligands interacting with transmembrane receptors (R) stimulate the enzyme adenylyl cyclase (AC) via interactions with G proteins (G). The subsequent rise in intracellular cAMP concentrations results in the dissociation of the regulatory and catalytic subunits of PKA, and the translocation of active catalytic subunits of PKA into the nucleus. PKA phosphorylates and thereby stimulates transcriptional activators, such as CREB, binding to CREs which induce transcription from the promoters of cAMP-responsive genes.

Although the precise mechanisms underlying cAMP-mediated regulation of granulocyte apoptosis are unknown, we have demonstrated clearly that (a) elevation of cAMP, either by cell permeable analogs or by PG receptor-directed agonists, inhibits neutrophil and eosinophil apoptosis, (b) cAMP-induced inhibition of neutrophil apoptosis, but not eosinophil apoptosis, is mediated by a PKA-dependent mechanism, (c) dexamethasone-mediated effects on neutrophil apoptosis, but not eosinophil apoptosis, may also be regulated by a PKA-dependent mechanism, (d) LPS and elevated $[Ca^{2+}]_i$-induced suppression of neutrophil apoptosis is not mediated by PKA activation, and importantly (e) glucocorticoids, via a steroid receptor-dependent mechanism, up-regulate the activity of a predominantly cytosolic PKA, which we have shown exists in two isoforms in human neutrophils. Therefore, I believe that this work has shed some light on the mechanisms regulating cAMP- and steroid-mediated effects on granulocyte apoptosis and lays the foundation for future work probing the possible interactions between glucocorticoid and PKA pathways.

Chapter 7

Effect of glucocorticoids on macrophage recognition of aged granulocytes

7.1 Introduction

The inflammatory macrophage plays a central role in the inflammatory process, being able to secrete and respond to a range of pro-inflammatory cytokines that mediate the initiation, progression and resolution of inflammation. Apoptosis is now emerging as an efficient mechanism for clearance of unwanted cells during the resolution of inflammation, without inciting any further inflammatory response (Savill & Haslett, 1995). Eosinophils (Stern et al., 1992) and neutrophils (Haslett et al., 1987) are constitutively programmed to undergo apoptosis which limits their pro-inflammatory potential and leads to rapid recognition by macrophages. Similar mechanisms have also been implicated in the clearance of lymphocytes (Pender et al., 1991) and monocytes (Mangan et al., 1991). It is thought that in mammals tissue macrophages are the key cells that remove apoptotic cells. However, cells of other lineages, such as glomerular mesangial cells (Savill et al., 1992a) and fibroblasts (Hall et al., 1994), may also act as 'semi-professional' phagocytes and assist in the clearance mechanism. Inflammatory infiltrates of eosinophils and neutrophils have been associated with the pathogenesis of a variety of diseases (Malech & Gallin, 1987), since these effector cells contain an array of agents, including cytotoxic and degradative enzymes, cationic proteins and inflammatory mediators, with the capacity to injure tissues and further exacerbate the inflammatory response (Henson & Johnston, 1987). Resolution of inflammation therefore requires rapid recognition of effete

cells to ensure against the detrimental consequences to the surrounding tissue, that would follow if cell disintegration or necrosis occurred. Neutrophil-mediated tissue damage, for example, has been implicated in the pathology of rheumatoid arthritis and adult respiratory distress syndrome (Weiss, 1989), whereas eosinophils have been specifically implicated in the pathogenesis of a number of diseases associated with a peripheral blood eosinophilia, including both allergy and asthma (Gleich et al., 1993). Nevertheless, most inflammatory reactions, including those associated with a massive granulocyte recruitment, resolve with return of normal tissue structure and organ function.

Initial studies providing direct support for an efficient mechanism of resolution, that might protect the host from incidental tissue injury, involved a series of *in vitro* experiments showing that senescent neutrophils can be engulfed by macrophages (Newman et al., 1982). These experiments were the first to highlight that neutrophils meet their fate *in situ*, where intact senescent neutrophils are removed by macrophages, providing an important injury-limiting neutrophil disposal route. It was subsequently clearly demonstrated that neutrophils had to be apoptotic before phagocytosis occurred since aged non-apoptotic neutrophils, as well as fresh neutrophils, are not recognised (Savill et al., 1989a). Further compelling evidence emphasizing the injury-limiting potential of neutrophil clearance by apoptosis, was provided by Meagher and colleagues, who have demonstrated that macrophages do not secrete pro-inflammatory thromboxane B_2 during phagocytosis of aged neutrophils, by contrast with the marked release of thromboxane B_2 which occurs in response to ingestion of opsonized erythrocytes or zymosan, which are standard 'control' particles (Meagher et al., 1992).

More recently, Bellingan et al., (1996) have investigated the *in vivo* fate of inflammatory macrophages during the resolution of inflammation and have demonstrated that these cells, in contrast to eosinophils and neutrophils, do not meet their fate locally by apoptosis and phagocytosis, but emigrate from the peritoneum specifically to draining lymph nodes.

Clearance of senescent or effete cells has been shown to play a vital role in many fundamental biological processes, including normal tissue turnover (Han et al., 1993) and remodelling of embryological tissues (Hopkinson-Woolley et al., 1994). Moreover, phagocytic clearance of apoptotic neutrophils by macrophages has been illustrated *in vivo* in inflammation of the joint (Savill et al., 1989a), lung (Grigg et al., 1991), gut (Savill, 1992) and kidney (Savill et al., 1992a). These observations strongly imply that cellular apoptosis and engulfment by macrophages provides a potential mechanism for the efficient clearance of redundant, intact inflammatory effector cells from inflamed tissue sites.

Given that glucocorticoids have been shown to influence macrophage function (Russo-Marie, 1992) and as we have shown in Chapter 3, that glucocorticoids profoundly and differentially regulate granulocyte apoptosis, we investigated the effect of dexamethasone on granulocyte clearance by macrophages *in vitro*.

7.2 Results

7.2.1 Effect of dexamethasone on macrophage phagocytosis of aged neutrophils

We have demonstrated that incubation of monocyte-derived macrophages with dexamethasone (1 μM) for 20 h, promoted macrophage recognition and phagocytosis of aged, apoptotic neutrophils (figure 7.1). In all experiments undertaken, neutrophils were aged in culture for 20 h, to ensure that the constitutive rate of apoptosis was sufficient (between 60-80% apoptosis) to allow a significant rate of phagocytosis by the control population of macrophages. Figure 7.2 illustrates photomicrographs of tissue culture plates bearing a monolayer of human monocyte-derived macrophages which have been interacted with aged, apoptotic neutrophils, before non-ingested neutrophils were washed away and the monolayer was fixed and stained for myeloperoxidase (MPO). Aged neutrophils are therefore identified as darkly stained circles within the MPO-negative macrophages. The interaction performed using dexamethasone (1 μM)-treated macrophages (figure 7.2*B*), demonstrates the dramatic increase in the proportion of macrophages ingesting aged, apoptotic neutrophils compared with the untreated population of macrophages in the control interaction (figure 7.2*A*). As illustrated in figure 7.2, the total number of macrophages per field was found to be consistent between control and dexamethasone-treated macrophages. These findings suggest that dexamethasone directly up-regulates phagocytosis of apoptotic cells, and is not due to a direct effect of dexamethasone inducing an increase in either the number of macrophages adhered to the tissue culture plate or the number of macrophages successfully differentiated from monocytes. A limitation of

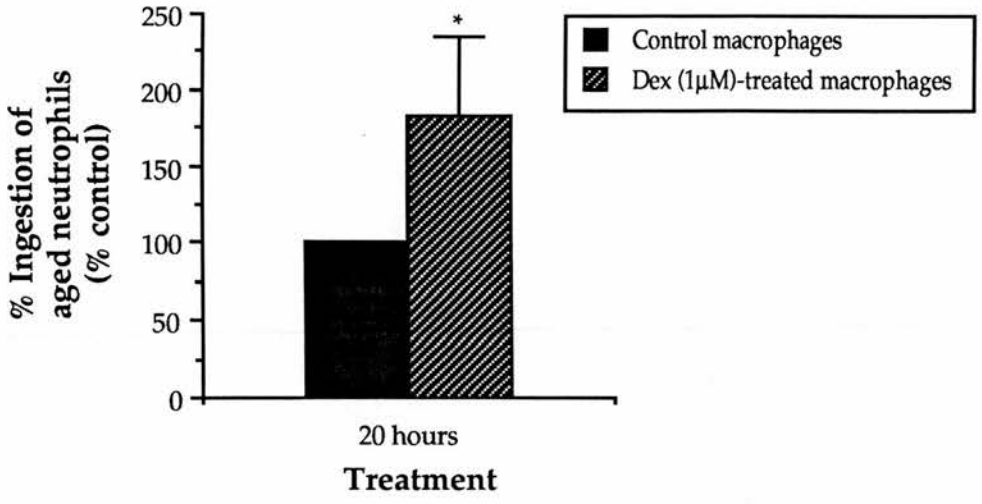

Figure 7.1 Effect of dexamethasone on macrophage recognition of aged neutrophils.
Monocyte-derived macrophages were either incubated alone or pre-incubated with dexamethasone (1 μM for 20 h), before washing and 30 min interaction with aged neutrophils. Data represent mean % of control ± SEM of 18 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values), in which under control conditions 37.2 ± 3.5% and in dexamethasone-treated macrophages, 61.7 ± 2.9% of macrophages ingested apoptotic neutrophils.

this *in vitro* method which is based on direct counting by light microscopy of macrophages which have ingested apoptotic cells, is that it is difficult to distinguish between binding and ingestion of the apoptotic cell by the phagocyte, although sufficient washing is performed to ensure that simply bound neutrophils are removed. However, attempts have been made to dissect the relative prevalence of these two processes by detaching the macrophages, following their interaction with aged neutrophils, from the plastic wells of the tissue culture plates and cytocentrifuging the cells onto a glass slide before staining. These studies (data not shown) have revealed that dexamethasone-mediated up-regulation of phagocytosis, promotes ingestion rather that binding of apoptotic neutrophils.

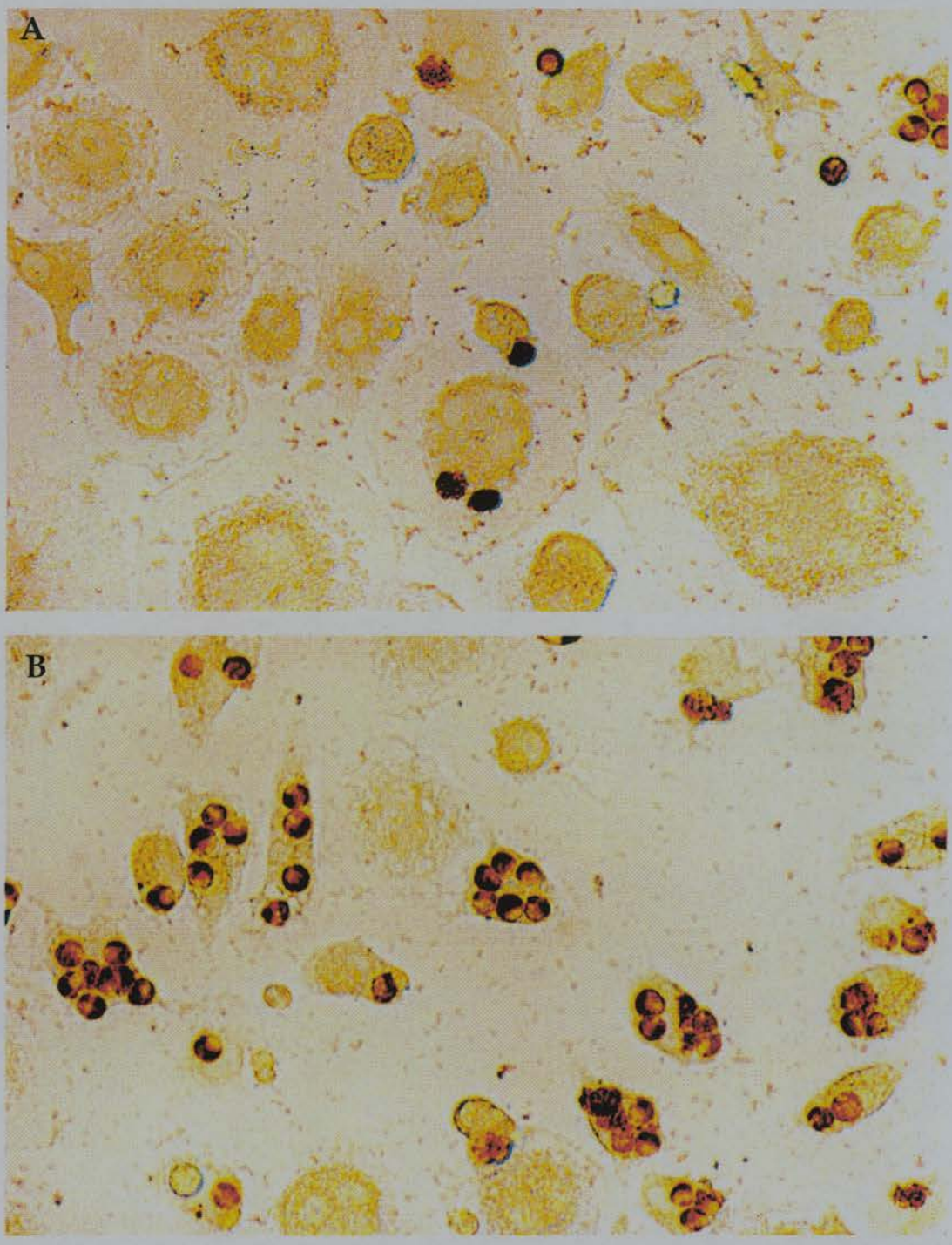

Figure 7.2 Effect of dexamethasone on macrophage recognition of aged neutrophils.

Photomicrographs (x1250 magnification) of the *in vitro* interaction between macrophages and apoptotic neutrophils. Monocyte-derived macrophages were incubated in the absence (*A*) and presence of dexamethasone (1 μM for 20 h) (*B*) before being overlaid with a suspension of apoptotic neutrophils for 30 min. Ingested neutrophils were stained for MPO. Note that macrophages are MPO-negative.

Moreover, we have also demonstrated that the endogenous glucocorticoid, cortisol, mirrors the effect of dexamethasone in potentiating macrophage recognition of apoptotic neutrophils. However, in contrast, progesterone, which has little glucocorticoid activity, does not augment macrophage recognition, as compared to control levels (figure 7.3).

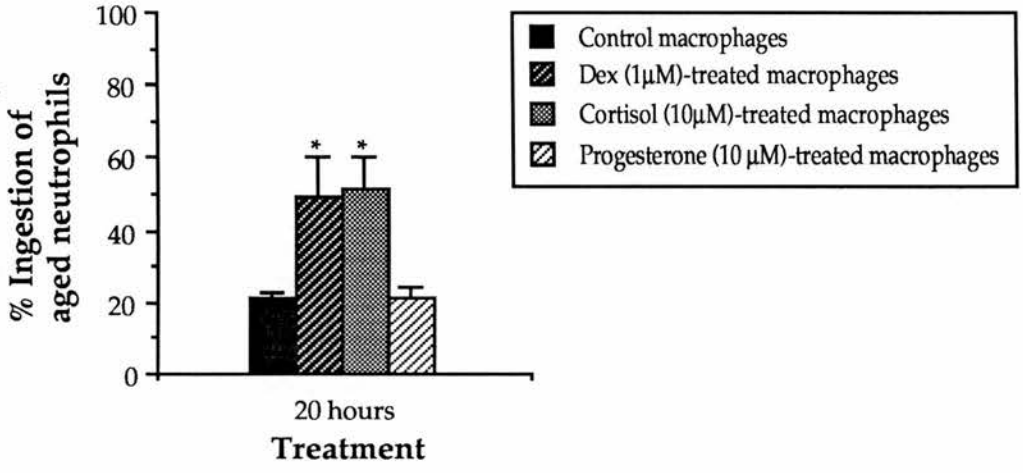

Figure 7.3 Effect of different corticosteroids on macrophage recognition of aged neutrophils.
Monocyte-derived macrophages were incubated in the absence and presence of dexamethasone (1 μM), cortisol (10 μM) and progesterone (10 μM) for 20 h, before being overlaid with a suspension of aged neutrophils. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

7.2.2 Effect of RU38486 on dexamethasone-mediated up-regulation of macrophage recognition of apoptotic neutrophils

Figure 7.4 illustrates that the glucocorticoid receptor antagonist, RU38486 (10 μM), while having no significant effect upon the control rate of macrophage recognition and ingestion, completely attenuated dexamethasone augmentation of macrophage recognition of apoptotic neutrophils. These data suggest that potentiation of macrophage recognition by dexamethasone is mediated via the glucocorticoid receptor.

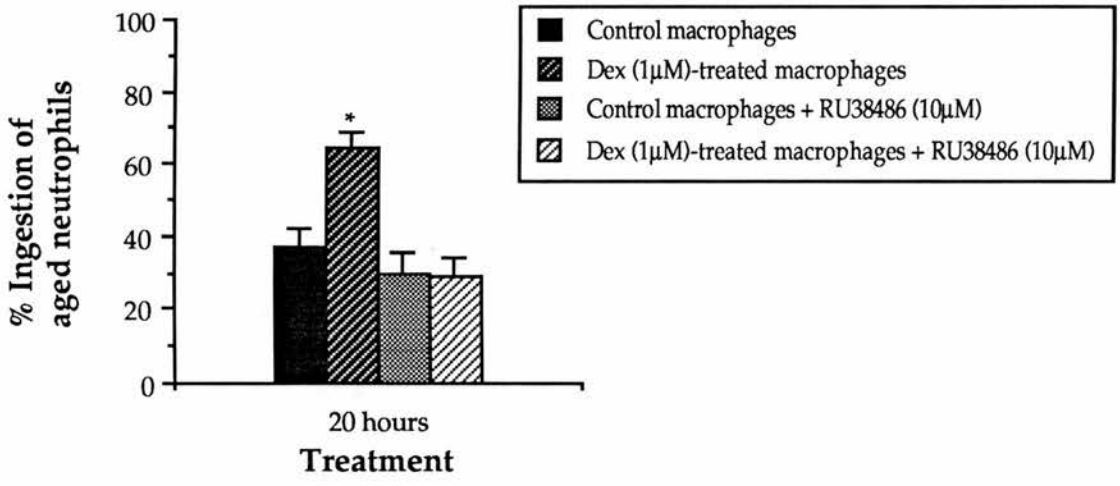

Figure 7.4 Effect of RU38486 on macrophage recognition of aged neutrophils.
Monocyte-derived macrophages were incubated either alone or with dexamethasone (1 μM for 20 h), in the absence and presence of RU38486 (10 μM for 20 h), before being overlaid with a suspension of aged neutrophils. Data represent mean ± SEM of 8 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

7.2.3 Time-course for the effect of dexamethasone on macrophage recognition of apoptotic neutrophils

Initial studies investigating the effect of dexamethasone on macrophage recognition of apoptotic neutrophils were performed using a pre-incubation time of 20 h, for the treatment of macrophages with dexamethasone (1 μM). We subsequently investigated the time-course of dexamethasone-mediated up-regulation of macrophage phagocytosis of apoptotic neutrophils.

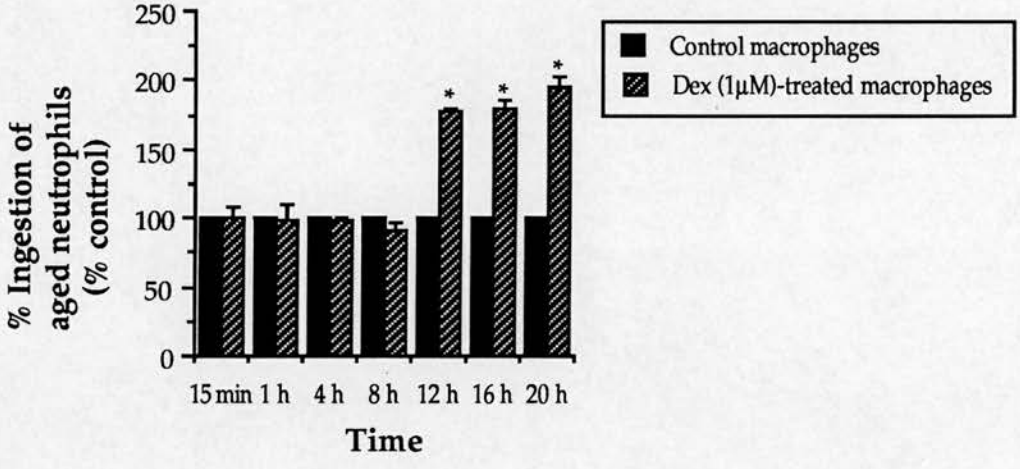

Figure 7.5 Time-course of the effect of dexamethasone on macrophage recognition of aged neutrophils.
Monocyte-derived macrophages were incubated in the absence and presence of dexamethasone (1 μM), for the time periods indicated, before being overlaid with a suspension of aged neutrophils. Data represent mean % of control ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values), in which under control conditions 10.9 ± 0.2% macrophages ingested apoptotic neutrophils.

Dexamethasone does not augment recognition of apoptotic neutrophils until macrophages have been pre-incubated for at least 12 h with dexamethasone before interaction with apoptotic neutrophils (figure 7.5). The potentiating effect of dexamethasone appears likely to be exerted on the macrophage and not the apoptotic neutrophil, since macrophages were washed prior to the interaction with apoptotic neutrophils and the degree of enhancement by dexamethasone (1 μM) depended on the time of incubation of macrophages with dexamethasone before interaction with apoptotic neutrophils. Thus potentiation was clearly apparent by 12 h, and maximal by 20 h (figure 7.5).

7.2.4 Concentration-dependency of the effect of dexamethasone on macrophage recognition of apoptotic neutrophils

We were interested to investigate the effect of increasing concentrations of dexamethasone (0.01-1 μM) on macrophage recognition of apoptotic neutrophils. Figure 7.6 illustrates that dexamethasone promotes recognition in a concentration-dependent manner, with an approximate EC_{50} value of 0.05 μM.

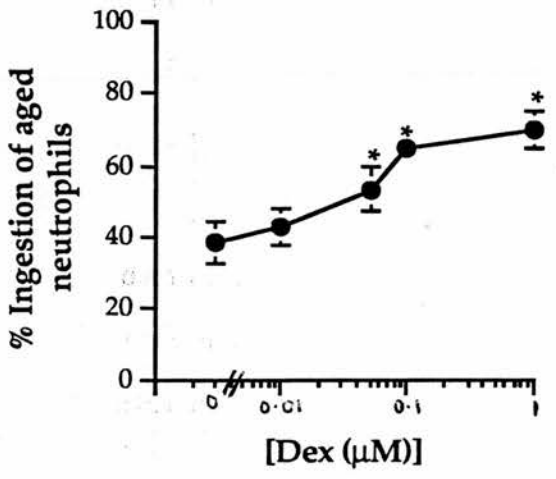

Figure 7.6 Concentration-response for the effect of dexamethasone on macrophage recognition of aged neutrophils.
Monocyte-derived macrophages were incubated in the absence and presence of dexamethasone (0.01-1 μM for 20 h), before being overlaid with a suspension of aged neutrophils. Data represent mean ± SEM of 7 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

7.2.5 Specificity of dexamethasone-mediated up-regulation of macrophage recognition

It has previously been demonstrated that macrophages selectively recognise and ingest intact neutrophils that have been aged in culture, however fresh neutrophils are not recognised (Newman et al., 1982, Savill et al., 1989a). These findings suggest that in the phagocytosis of apoptotic cells, the macrophage recruits a specific recognition mechanism. We were therefore interested to investigate whether dexamethasone-mediated up-regulation of apoptotic neutrophils, was selective to the

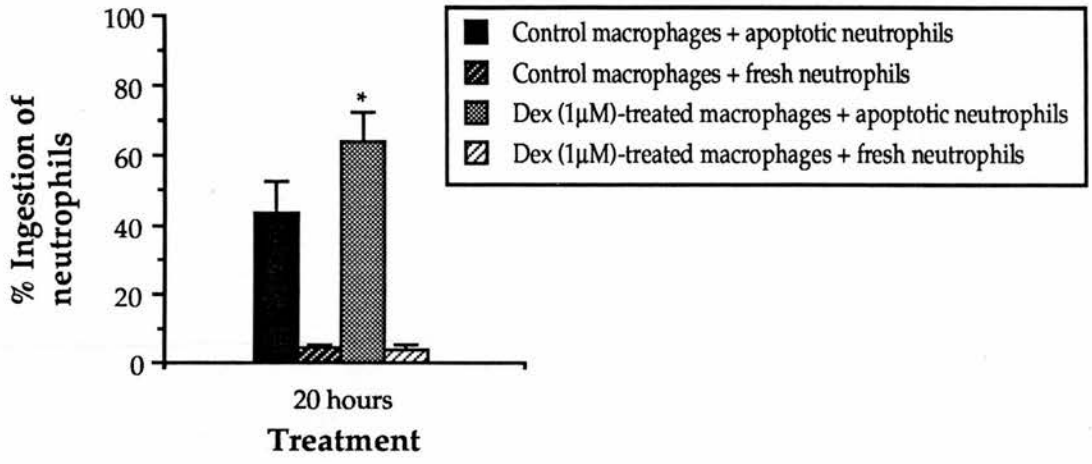

Figure 7.7 Comparison of the effect of dexamethasone on macrophage recognition of aged and fresh neutrophils.
Monocyte-derived macrophages were incubated in the absence and presence of dexamethasone (1 μM for 20 h) before being overlaid with a suspension of either apoptotic neutrophils, previously aged for 20 h in culture, or freshly isolated (non-apoptotic) neutrophils, for 30 min. Data represent mean ± SEM of 5 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

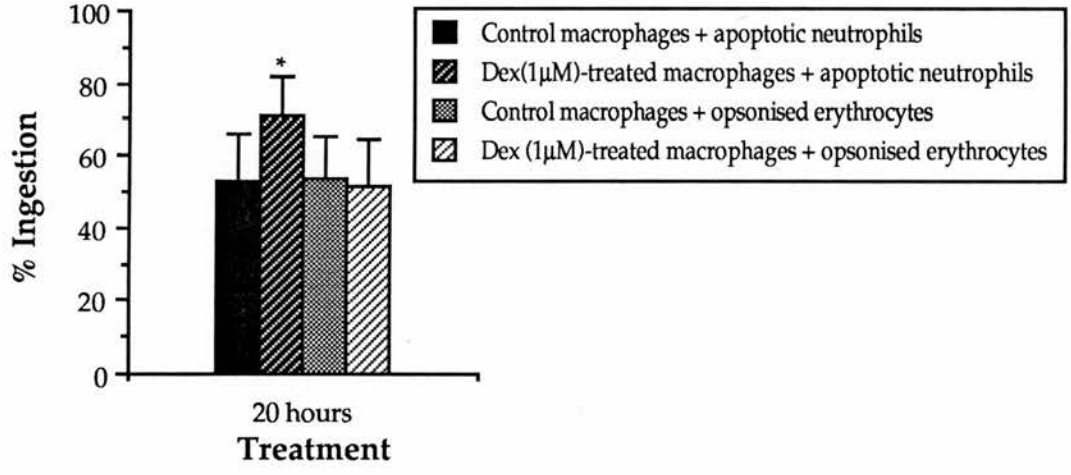

Figure 7.8 Comparison of the effect of dexamethasone on macrophage recognition of aged neutrophils and IgG-opsonised erythrocytes.
Monocyte-derived macrophages were incubated in the absence and presence of dexamethasone (1 μM for 20) before being overlaid with a suspension of either apoptotic neutrophils, previously aged for 20 h in culture, or IgG-opsonised erythrocytes, for 30 min. Data represent mean ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values).

phagocytosis mechanism employed for recognition of apoptotic cells or whether dexamethasone up-regulated an altogether non-specific stimulation of the phagocytic potential of macrophages. Figure 7.7 illustrates the effect of dexamethasone on macrophage recognition and ingestion of freshly isolated neutrophils (non-apoptotic) compared with aged, apoptotic neutrophils. These data demonstrate that both control and dexamethasone-treated macrophages did not phagocytose fresh neutrophils, whereas in the same experiment, apoptotic neutrophils were ingested. Moreover, dexamethasone did not augment macrophage recognition of immunoglobulin-opsonised erythrocytes (the standard 'control' particle) as compared to control macrophages (figure 7.8), whereas in the same experiment, aged, apoptotic neutrophils were ingested by control macrophages and this phagocytic capacity was shown to be increased in dexamethasone-treated macrophages. Collectively, these data exclude the possibility that dexamethasone up-regulates non-specific phagocytosis by macrophages and suggest that dexamethasone may have an important role in promoting the efficient clearance of apoptotic cells, thereby promoting the resolution of inflammation.

7.2.6 Effect of dexamethasone on macrophage phagocytosis of aged eosinophils

Interestingly, we also demonstrated that incubation of monocyte-derived macrophages with dexamethasone (1 μM) for 20 h, promoted macrophage recognition and phagocytosis of aged, apoptotic eosinophils (figure 7.9). In the preliminary experiments undertaken, eosinophils were aged for 40 h in culture, to ensure that the constitutive rate of apoptosis, which is slower in eosinophils compared to neutrophils, was sufficient to allow a significant rate of phagocytosis by the control population of macrophages.

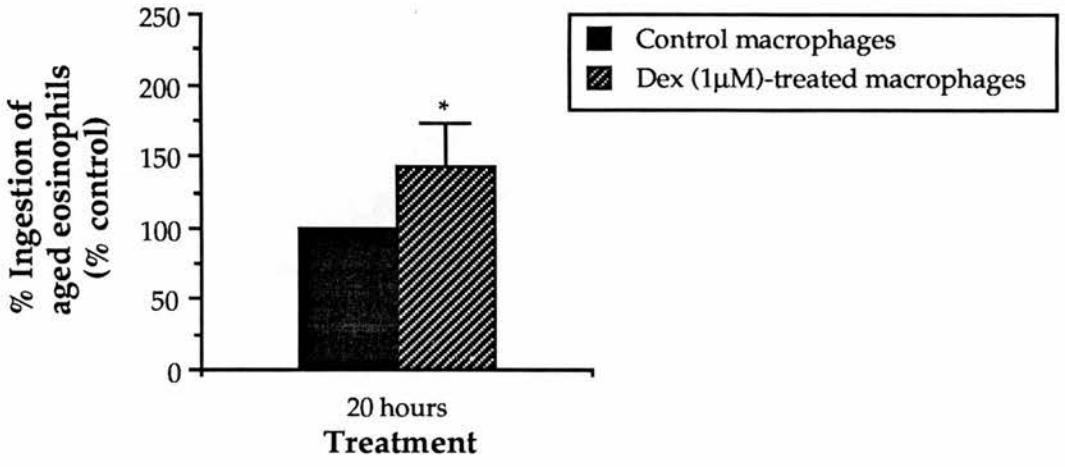

Figure 7.9 Effect of dexamethasone on macrophage recognition of aged eosinophils.
Monocyte-derived macrophages were either incubated alone or pre-incubated with dexamethasone (1 μM for 20 h), before washing and 30 min interaction with aged eosinophils. Data represent mean % of control ± SEM of 3 separate experiments, each performed in duplicate (* $p<0.05$ compared with control values), in which under control conditions 71.5 ± 13.3% and in dexamethasone-treated macrophages, 94.0 ± 1.1% of macrophages ingested apoptotic eosinophils.

7.3 Discussion

In this study, we have examined whether glucocorticoids modulate human macrophage phagocytosis of senescent eosinophils and neutrophils undergoing apoptosis. We have demonstrated that macrophages pre-treated with dexamethasone (1 μM) for 20 h prior to interaction with aged, apoptotic granulocytes, showed an augmentation in their phagocytic capacity as compared to control, untreated macrophages (figures 7.1 & 7.9). This is a novel and particularly interesting finding in view of the common use of glucocorticoids as potent anti-inflammatory agents. These results suggest that in addition to modulating granulocyte apoptosis (Chapter 3), dexamethasone may also regulate the clearance of aged, apoptotic cells, an event which may promote more efficient resolution of the inflammatory response.

Dexamethasone-mediated regulation of granulocyte clearance is particularly exciting in view of the differential effect of glucocorticoids upon granulocyte apoptosis, discussed in Chapter 3. It is therefore interesting to speculate about the *in vivo* significance of why dexamethasone should augment eosinophil apoptosis and inhibit neutrophil apoptosis, yet promote the clearance of both cell types. This observation may be related to the kinetics of effector cell arrival at an inflammatory site. Neutrophils are the first cells to arrive at the scene of tissue perturbation and as such it may be desirable that the effective life of the extravasated neutrophil is prolonged in order to fight invading microbes, thereby promoting host defence. Moreover, this may allow time for recruited monocytes, which are unable to ingest apoptotic neutrophils, to differentiate into phagocytically competent macrophages (Savill et al., 1990).

However, when these granulocytes eventually succumb to apoptosis, glucocorticoids enhance their removal by macrophages. The eosinophil, however, in allergic reactions and diseases is largely an unwanted cell, therefore glucocorticoids may induce their apoptosis and subsequent removal accordingly. The differential effect of dexamethasone on granulocyte apoptosis *in vitro,* may therefore co-operate with the temporal recruitment of granulocytes at an inflammatory site. By promoting apoptotic clearance, dexamethasone facilitates ingestion of intact granulocytes without disgorgement of their toxic contents, further ensuring an alternative fate to necrosis and thereby promoting the normal resolution process of inflammation. Collectively these findings may give rise to new anti-inflammatory treatments, as evidenced by clearance of eosinophils from a model of allergic lung inflammation (Tsuyuki et al., 1995) by intratracheal instillation of agents ligating Fas on murine lung eosinophils and thereby inducing apoptosis and phagocytic clearance. Tsuyuki and colleagues have demonstrated that macrophage-mediated phagocytosis of eosinophils, as revealed by an increase in the number of peroxidase positive macrophages, occurred *in vivo,* after treatment with the anti-Fas mAb and was temporarily associated with the resolution of eosinophilic inflammation. Interestingly, this report shows that the increase in the number of peroxidase positive macrophages was transient; a finding supported by *in vitro* observations, demonstrating that when neutrophils are ingested by macrophages, they are degraded within 30 min and are no longer recognisable (Savill et al., 1989a). Moreover, Tsuyuki et al., (1995) also demonstrated that 24-48 h after mAb treatment, the number of peroxidase positive macrophages increased compared to time 0 (i.e., 72 h after antigen challenge), and suggest that under control conditions, eosinophils undergo apoptosis and are

engulfed by macrophages as the 'normal surveillance' mechanism to remove unwanted apoptotic cells.

Similar findings have also been reported in neutrophilic inflammatory models, *in vivo*. Cox and colleagues used a rat model of acute pulmonary inflammation induced by intratracheal administration of LPS, which induces a prompt marked neutrophilic response in the lung that peaks at 18 h and resolves after 72 h (Cox et al., 1995). This study showed that the neutrophilic response was accompanied by the presence of a gradually increasing number of apoptotic neutrophils and of macrophages containing apoptotic neutrophils, peaking at 24 h, and declining with resolution of the neutrophilic response. These observations demonstrate that macrophage-mediated engulfment of apoptotic neutrophils after an acute inflammatory response is both temporally correlated with and required for the resolution of acute neutrophilic inflammation. Moreover, there have been two reports, particularly germane to our *in vitro* findings, of the potentiation of macrophage clearance of apoptotic granulocytes by dexamethasone. It has been shown *in vivo* that macrophages engulf eosinophils with apoptotic morphology in the jejunal mucosa of parasitized rats treated with dexamethasone (Kawabori et al., 1991) and more recently Woolley and colleagues have provided the first report of eosinophil apoptosis *in vivo* in human asthma (Woolley et al., 1996). The latter report showed that eosinophil apoptosis increased after corticosteroid treatment for an exacerbation of asthma and that eosinophil products were apparent within macrophages. The increase in eosinophil apoptosis occurred in association with clinical improvement and the resolution of eosinophilic inflammation. Taken together with

our *in vitro* findings, these results suggest that apoptosis is a mechanism for the reduction of eosinophilic airway inflammation in asthma.

Interestingly, eosinophil and neutrophil apoptosis can be slowed down by a number of factors, including activating agents such as LPS and the cytokine GM-CSF (Squier et al., 1995, Lopez et al., 1986, Takanaski et al., 1994). Moreover, it has previously been reported that GM-CSF and other pro-inflammatory cytokines increased phagocytosis of apoptotic neutrophils by a rapid and selective effect on the macrophage (Ren & Savill, 1995). This report suggested that GM-CSF may act in a coordinate fashion on neutrophils and monocyte-derived macrophages to ensure that apoptosis in the former is carefully timed to match acquisition of phagocytic capacity by the latter. These suggestions mirror the temporal explanations offered to account for the differential effect of dexamethasone on granulocyte apoptosis and promotion of macrophage clearance of both cell types in our system.

We have demonstrated that dexamethasone-induced up-regulation of phagocytosis is limited to glucocorticoids (figure 7.3) and is time- (figure 7.5) and concentration-dependent (figure 7.6). Moreover, dexamethasone appears to selectively up-regulate the phagocytic mechanism employed for recognition of apoptotic cells, rather than a wholly non-specific stimulation of the phagocytic capacity of macrophages (figures 7.7 and 7.8). These findings suggest that dexamethasone-treated macrophages, like untreated (control) phagocytes, can recognise changes on the surface of cells as they undergo apoptosis which identifies them as 'senescent self'. Furthermore, the glucocorticoid receptor antagonist, RU38486, was shown to inhibit potentiation of macrophage recognition by dexamethasone,

while having no effect on the control rate of macrophage phagocytosis (figure 7.4). These results suggest the involvement of macrophage glucocorticoid receptors, which have been previously identified as high affinity receptors (Werb et al., 1978), as having an important role in mediating the effect of dexamethasone on phagocytosis. Further experiments are required to determine the precise mechanism(s) underlying dexamethasone-mediated potentiation of phagocytic clearance of apoptotic granulocytes. However, over recent years there has been intense interest in the mechanisms by which phagocytes recognise cells undergoing apoptosis *in vitro*, and some of these now elucidated mechanisms may also be implicated in dexamethasone-mediated up-regulation of granulocyte clearance. The known molecular mechanisms underlying the recognition of apoptotic cells are summarised in table 7.1 (Savill et al., 1993, Hart et al., 1996, Savill, 1997). Significantly, eosinophil (Stern et al., 1996) and neutrophil (Savill et al., 1992b) data indicate that phagocytosis depends upon two macrophage receptors for TSP, the $\alpha_v\beta_3$ vitronectin receptor integrin and CD36 (see figure 7.10). Antibody inhibition experiments have now shown that this $\alpha_v\beta_3$/TSP/CD36 recognition mechanism is also employed by macrophage uptake of apoptotic lymphocytes (Akbar et al., 1994). It is therefore possible that since monocyte-derived macrophages ingest both apoptotic eosinophils and neutrophils by deploying the $\alpha_v\beta_3$/TSP/CD36 recognition mechanism, that dexamethasone-mediates potentiation of phagocytosis by increasing the expression of these macrophage surface receptors and also macrophage secretion of TSP. However, further experiments are necessary to determine the precise mechanism by which dexamethasone augments phagocytosis of apoptotic granulocytes.

Molecular mechanisms of recognition of apoptotic cells by phagocytes	References
Lectin-like receptors Specific carbohydrates on the surface of apoptotic cells are recognised by phagocytic cells containing lectin-like receptors such as the asialoglycoprotein, mannose or mannose/fructose receptor.	Duvall et al., 1985, Hall et al., 1994., Dini et al., 1992, Dini et al., 1995.
$\alpha_v\beta_3$/TSP/CD36 It is hypothesized that a TSP binding moiety expressed on the surface of apoptotic cells binds to nearby TSP which in turn acts as a bridging molecule between the apoptotic cell and the ingesting phagocyte. The phagocyte expresses two receptors on its surface; the $\alpha_v\beta_3$ integrin or 'vitronectin receptor' and CD36, which co-operate to bind TSP.	Savill et al., 1989b, Savill et al., 1990, Savill et al., 1992b, Ren et al., 1995, Ren & Savill, 1995.
Phosphatidylserine (PS) and PS receptors Exposure of phosphatidylserine on the surface of apoptotic cells is believed to be recognised by putative phosphatidylserine receptors located on the surface of the ingesting phagocyte. Recently, it has been suggested that members of the scavenger receptor family may act as PS receptors.	Fadok et al., 1992a, Fadok et al., 1992b, Fadok et al., 1993, Martin et al., 1995, Verhoven et al., 1995, Platt et al., 1996, Fukasawa et al., 1996, Sambrano & Steinberg, 1995.
61D3 antigen The mAb 61D3 can specifically attenuate the recognition of apoptotic cells by human monocyte-derived macrophages. The 61D3 antigen has not been fully characterised.	Flora & Gregory, 1994.

Table 7.1 Summary of recognition mechanisms of apoptotic cells identified todate.

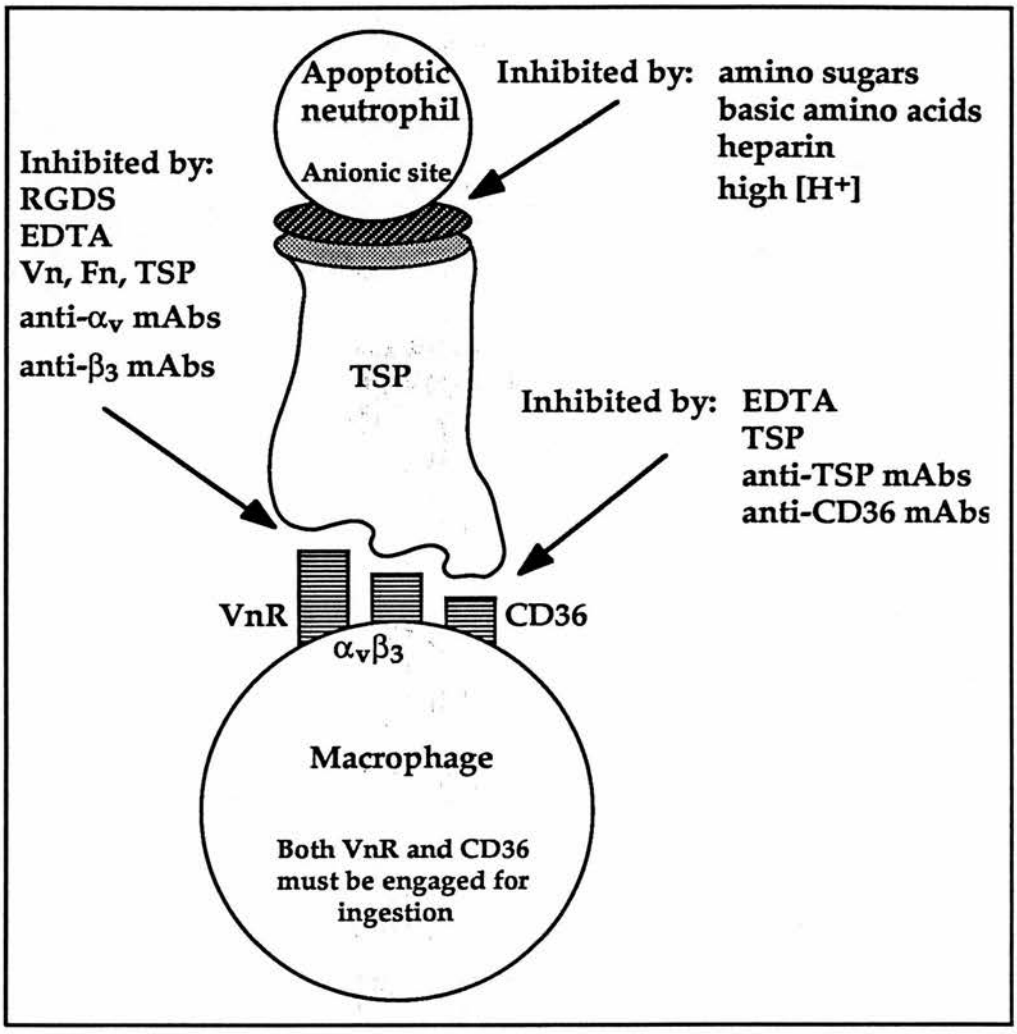

Figure 7.10 Diagram illustrating $\alpha_v\beta_3$/TSP/CD36 phagocytic recognition mechanism of apoptotic neutrophils. Abbreviations: Vn, vitronectin; Fn, fibronectin; VnR, $\alpha_v\beta_3$, vitronectin receptor integrin; TSP, thrombospondin. (Diagram from Savill, 1992).

In summary, we have demonstrated that dexamethasone promotes macrophage recognition and ingestion of aged, apoptotic granulocytes *in vitro*. This finding is particularly exciting in view of the recent *in vivo* reports discussed, and may potentially underlie some of the anti-inflammatory mechanisms employed by glucocorticoids, in mediating the temporal correlation of granulocyte clearance with the resolution of acute eosinophilic and neutrophilic inflammation. Recent findings by Stern and colleagues demonstrating that uptake of post-apoptotic eosinophil fragments increases macrophage release of pro-inflammatory mediators (Stern et al., 1996), accentuates the significance of our findings with regards to the resolution of inflammation.

The mechanism by which dexamethasone selectively potentiates recognition and phagocytosis of apoptotic cells has not been determined. However, we have highlighted several recognition mechanisms that are known to be deployed by macrophages in the clearance of apoptotic granulocytes and other cells. Dexamethasone may therefore increase the expression of selective macrophage surface receptors such as CD36 or alternatively may recruit its own novel and specific mechanism mediating the clearance of apoptotic cells. Further experiments are essential to establish any firm conclusions regarding the phagocytic mechanism of dexamethasone-mediated up-regulation of apoptotic granulocytes.

Interestingly, our laboratory has recently reported that ligation of the cell surface glycoprotein CD44 on macrophages, causes a specific augmentation of the phagocytosis of apoptotic neutrophils, but not of apoptotic lymphocytes. However, as with dexamethasone-mediated

upregulation of phagocytosis, the precise mechanism underlying CD44-augmentation of phagocytosis, has not been clearly defined (Hart et al., 1997).

In conclusion, our findings may result in conferring an anti-inflammatory advantage upon tissues, providing the possibility of therapeutic gain, by modulating the inflammatory response such that resolution is favoured rather than tissue destruction and fibrosis.

Chapter 8

Summary and General Discussion

We have demonstrated that glucocorticoids differentially affect the rate of granulocyte apoptosis *in vitro*; promoting eosinophil apoptosis and inhibiting neutrophil apoptosis. Dexamethasone-induced modulation of granulocyte apoptosis is limited to glucocorticoids and is specifically modulated via the glucocorticoid receptor. Moreover, the diametrically opposed effects of dexamethasone on granulocyte apoptosis does not appear to result from the differential elaboration of GM-CSF by eosinophils and neutrophils. Therefore, these results are particularly intriguing since they suggest that the apoptotic programme is under different controls in these two closely related myeloid cells. Hence interest has been focussed on dissecting the intracellular signalling processes whereby this discrepancy occurs.

We have presented data implicating both Ca^{2+} and PKC in the regulation of granulocyte apoptosis. Elevation of $[Ca^{2+}]_i$ using A23187, up-regulates eosinophil apoptosis. In contrast, elevation of $[Ca^{2+}]_i$ through independent mechanisms, by using either A23187 or thapsigargin, down-regulates neutrophil apoptosis. Thus, the effect of Ca^{2+} elevation on granulocyte apoptosis appears to mirror the effects of dexamethasone; promoting eosinophil apoptosis and inhibiting neutrophil apoptosis. However, experiments designed to investigate the role of Ca^{2+} in dexamethasone-mediated inhibition of neutrophil apoptosis have indicated that Ca^{2+} may not be the intracellular second messenger involved. Direct measurements of cytosolic Ca^{2+} levels in freshly isolated

neutrophils did not identify any changes in the $[Ca^{2+}]_i$ above resting values upon addition of dexamethasone. Moreover, BAPTA/AM, an intracellular Ca^{2+} chelator, did not modulate dexamethasone-mediated inhibition of neutrophil apoptosis. Thus, preliminary studies argue against a role for calcium in dexamethasone-mediated neutrophil apoptosis. We have also demonstrated that treatment of both eosinophils and neutrophils with agents that inhibit PKC induces apoptosis, thereby indirectly implicating a role for PKC in the suppression of granulocyte apoptosis. Moreover, at a concentration found to have no significant effect upon the rate of constitutive apoptosis, the PKC inhibitors, staurosporine and Ro-31-8220, partially abrogated dexamethasone-mediated inhibition of neutrophil apoptosis. Thus, activation of PKC may, in part, underlie the anti-inflammatory action of glucocorticoids.

With the advent of PD 098059, which is a selective inhibitor of the MAP kinase-activating enzyme MEK and hence the MAPK/ERK cascade, we have been able to study the involvement of MAPK/ERK in the regulation of granulocyte apoptosis. We have demonstrated that inhibition of the MAPK/ERK pathway down-regulates the basal rate of eosinophil apoptosis, while having no effect on the basal rate of neutrophil apoptosis. Thus, these data may suggest that activation of this signalling cascade may be an important component promoting eosinophil apoptosis and thereby facilitate the resolution of inflammation. Moreover, PD 098059 reversed LPS-mediated inhibition of neutrophil apoptosis while having no effect on glucocorticoid-induced suppression of neutrophil apoptosis. Thus, the effect of glucocorticoids on the rate of neutrophil apoptosis does not appear to involve MAPKs/ERKs.

Since the level of protein phosphorylation in any cell ultimately depends upon the combined activities of both protein kinases and protein phosphatases we were interested to investigate the effect of protein phosphatase inhibitors on the rate of granulocyte apoptosis. We have demonstrated that okadaic acid has a bi-phasic effect upon the rate of granulocyte apoptosis; with lower concentrations inhibiting apoptosis and higher concentrations promoting apoptosis. These results may reflect the ability of okadaic acid to differentiate between PP2A and PP1, promoting selective inhibition depending on the concentration used. Thus, at lower concentrations okadaic acid preferentially inhibits PP2A, whereas at higher concentrations okadaic acid inhibits both PP2A and PP1 in concert. These data may therefore suggest that the specific inhibition of PP2A regulates inhibition of granulocyte apoptosis, whereas the collective inhibition of both PP2A and PP1 is responsible for the promotion of apoptosis in these granulocytes. Moreover, at a concentration found to have no significant effect on the rate of granulocyte apoptosis, okadaic acid partially attenuated the pro-apoptotic effect of dexamethasone on eosinophils and completely attenuated dexamethasone-mediated inhibition of neutrophil apoptosis. Thus, these results suggest that glucocorticoid-mediated granulocyte apoptosis requires the activity of protein phosphatases.

We have observed that agents that elevate intracellular levels of cAMP inhibit granulocyte apoptosis. Interestingly, H-89, a selective inhibitor of PKA, does not modulate the rate of constitutive granulocyte apoptosis suggesting that PKA may not be important in the regulation of basal granulocyte apoptosis. However, cAMP-induced inhibition of neutrophil apoptosis, in contrast to eosinophil apoptosis, does appear to be mediated

by a PKA-dependent mechanism. Similarly, dexamethasone-mediated effects on neutrophil apoptosis, but not eosinophil apoptosis, also appear to be regulated by a PKA-dependent mechanism. Significantly, direct measurement of PKA activity after dexamethasone treatment in neutrophils revealed increased activity compared to the control population of cells, of a predominantly cytosolic localised PKA, which we have shown exists in two isoforms.

As discussed in Chapter 5, the finding that protein phosphatase inhibition and PKA inhibition both individually attenuate dexamethasone-mediated inhibition of neutrophil apoptosis appears confusing. However, these results may suggest that the protein phosphatase inhibitors, okadaic acid and calyculin A, phosphorylate different substrate proteins to those phosphorylated by PKA, hence providing an explanation as to why both protein phosphorylation and dephosphorylation result in modulation of dexamethasone-mediated inhibition of neutrophil apoptosis.

Finally, we have demonstrated that dexamethasone promotes macrophage-mediated phagocytosis of aged, apoptotic granulocytes *in vitro*. More detailed studies on the recognition and ingestion of aged neutrophils by macrophages, have revealed that dexamethasone induced up-regulation of phagocytosis is limited to glucocorticoids and is specifically mediated via the glucocorticoid receptor. Moreover, dexamethasone appears to selectively up-regulate the phagocytic mechanism employed for recognition of apoptotic cells rather than a wholly non-specific stimulation of the phagocytic capacity of macrophages. These findings are particularly exciting and may

potentially underlie the anti-inflammatory mechanisms used by glucocorticoids, in facilitating the clearance of unwanted inflammatory cells. These data are particularly germane in light of recent *in vivo* reports demonstrating the temporal correlation of granulocyte clearance with the resolution of acute eosinophilic and neutrophilic inflammation after glucocorticoid administration (Cox et al., 1995, Woolley et al., 1996).

Thus, using a pharmacological approach, many insights have been made into the intracellular signalling pathways regulating constitutive and glucocorticoid-mediated granulocyte apoptosis. Our observations do have limitations, however, in view of the unknown precise specificity of the agents used in our cellular system. New and more specific pharmacological tools may be useful to probe our findings further in the future. Since peripheral blood eosinophils are relatively low and comparatively more difficult to isolate, the majority of the investigations featured in this thesis have been undertaken on the neutrophil. However, preliminary and comparative studies on the eosinophil, have highlighted some important differences between the regulation of apoptosis in these two closely related myeloid cells. Thus, there seems to be a fundamental inter-relationship between the regulation of granulocyte apoptosis and signal transduction. While we have focussed on investigating the individual influences, upon granulocyte apoptosis, of modulating specific intracellular pathways, it is important to remember that there is a multitude of diverse interactions between single signalling pathways. These interactions include potentiation, co-operation, synergism and antagonism; many examples of which have been addressed during the course of this thesis (figure 8.1). Thus, at this stage of our understanding it is difficult to tie all of these apparently

disparate pieces of data into one comprehensive picture. However, despite these alternative signalling pathways, at some point it is likely that the signals converge into a common step or steps that allow progression of the apoptotic programme.

Given that in chronic inflammation there is a persistent accumulation of inflammatory cells which are likely to liberate their armamentarium of noxious intracellular products leading to tissue injury and scarring, it seems likely that effective removal of these potentially tissue damaging inflammatory cells would be desirable. Apoptosis therefore provides a mechanism for the safe disposal of extravasated granulocytes as the inflammatory response evolves. Significantly, differences between the intracellular regulation of inflammatory cells provide a special opportunity to selectively induce apoptosis in discrete populations of inflammatory cells, for which it may be possible to extrapolate these findings for therapeutic gain. The anti-inflammatory action of glucocorticoids is thought to be a crucial component of their efficacy in allergic diseases. However, this is tempered by the side-effects of these drugs, which has fuelled the desire to create compounds that have steroid-like actions but fewer side-effects. In combination with an effective therapeutic strategy to selectively induce apoptosis, development of mechanisms to specifically enhance apoptotic cell clearance by phagocytes, such as the macrophage, may be equally desirable in promoting the resolution of inflammation.

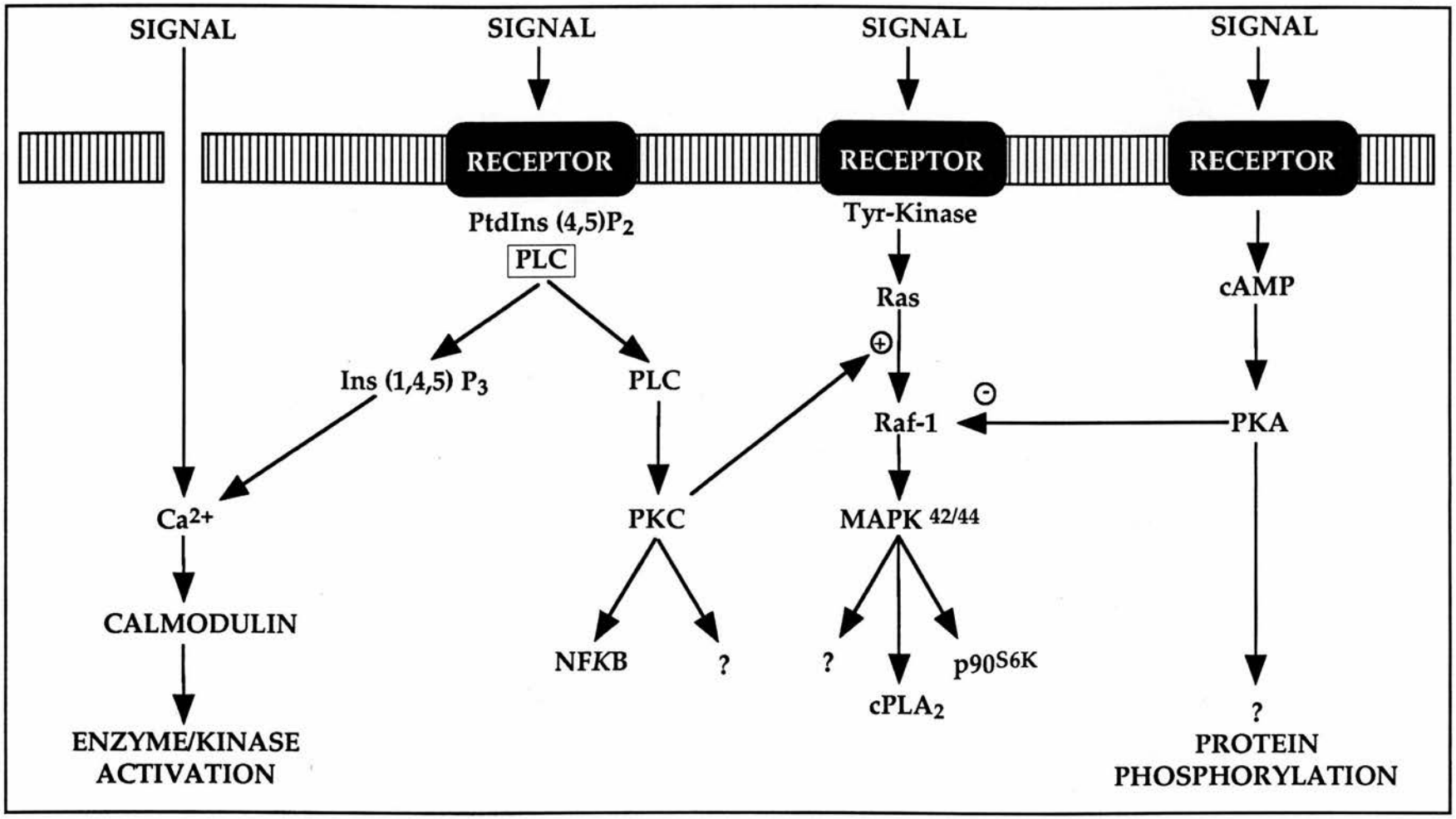

Figure 8.1 Summary of the signal transduction pathways possibly involved in the regulation of granulocyte survival.

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APPENDIX

A summary of the main eosinophil and neutrophil granules and secretory vesicles.

EOSINOPHIL		
PRIMARY GRANULES	**SECONDARY GRANULES**	**SMALL GRANULES**
No crystalloid core Lysophospholipases ECP EDN	Crystalloid core Composed of MBP Surrounding matrix containing ECP, EDN and EPO	Aryl-sulphatase

NEUTROPHIL			
AZUROPHIL GRANULES	**SPECIFIC GRANULES**	**GELATINASE GRANULES**	**SECRETORY GRANULES**
Defensins Myeloperoxidase Lysozyme Acid hydrolases	Lactoferrin Cytochrome b_{558} fMLP-R CD11b Lysozyme Collagenase Vitamin B_{12}-binding	Gelatinase	Alkaline phosphatases CD11b fMLP-R Cytochrome b_{558}